Global assessment of host cell functions involved in the intracellular survival and replication of Chlamydia using RNA interference in human cells

Dissertation

zur Erlangung des Akademischen Grades

doctor rerum naturalium

(Dr. rer. nat.)

im Fach Biologie

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät I

Humboldt-Universität zu Berlin

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Tag der mündlichen Prüfung: 14. Juli 2009

ZUSAMMENFASSUNG

Chlamydia trachomatis ist ein obligat intrazellulär lebendes, gram-negatives Bakterium. Es wird mit einer Vielzahl von Krankheiten in Verbindung gebracht, darunter vermeidbare Blindheit (Trachom) und reaktive Arthritis. Zudem ist es der häufigste bakterielle Auslöser sexuell übertragener Krankheiten, mit für Frauen häufig fatalen Folgen wie Eileiterentzündung, Extrauterinschwangerschaft und Sterilität. Als obligat intrazelluläres Pathogen hängt Chlamydia trachomatis in nahezu allen Aspekten seines Überlebens von der Wirtszelle ab, angefangen bei der Anheftung an die Wirtszelle, über die Invasion, der Aufnahme von Wirtszellmetaboliten bis zur intrazellulären Replikation. Intrazelluläre Interaktionen zwischen Wirt und Pathogen spielen bei der erfolgreichen Chlamydien-Infektion eine entscheidende Rolle. Trotz der weiten Verbreitung und der immensen Bedeutung bei verschiedenen Krankheiten ist wenig über die dem Infektionsprozess und den der Chlamydien-Replikation zugrundeliegenden Mechanismen bekannt. Für ein vollständiges Bild der Pathogenese sind sowohl das Verstehen der dazu beitragenden bakteriellen wie auch Wirtszellfaktoren essenziell. Die vorliegende Arbeit konzentriert sich dabei auf die an der Infektion beteiligten Wirtsfaktoren. Für derartige Studien hat sich das Ausschalten von Genfunktionen mittels RNA-Interferenz als ein robustes und zielgerichtetes Werkzeug herausgestellt. Sie ist die Methode der Wahl für sogenannte Funktionsverluststudien (loss-of-function studies) und kann dazu dienen, den Beitrag der Wirtszelle an der Chlamydien-induzierten Pathogenese aufzuklären.

Um tiefere Einblicke in die Abläufe chlamydialer Infektionen zu erlangen, wurde in dieser Arbeit ein RNA-Interferenz-vermittelter Funktionsverlustscreen durchgeführt. Als Indikator für Effekte auf die primäre Infektion wurde die Anzahl chlamydialer Inklusionen sowie deren Größe bestimmt. Um Aufschlüsse über den Einfluss von Wirtszellfaktoren für den kompletten Durchlauf des bakteriellen Lebenszyklus zu erlangen wurde zudem die Chlamydien-Vermehrung quantifiziert. In diesem Rahmen wurden 1500 humane Gene, die unter anderem das gesamte humane Kinom, sowie für Apoptose und für den intrazellulären Transport relevante Gene umfassten, durchmustert. Mithilfe verschiedener statistischer Methoden wurden 132 primäre Hits selektioniert. Um mögliche, bei der RNA-Interferenz auftretende, zielgenunabhängige Effekte auszuschließen, die zu falsch-positiven Hits führen

können, wurden die primären Hits mit vier weiteren siRNAs validiert. Damit gelang die Identifizierung von 59, für die Chlamydien-Infektion und Vermehrung relevanten Genen.

Unter Zuhilfenahme von bioinformatischen Signaltransduktionsweganalyse-programmen konnten einige der Hits bekannten zellulären Signalnetzwerken, unter anderem dem Ras/Raf/Mek/Erk-Signalweg, zugeordnet werden. Insbesondere der Funktionsverlust zweier validierter Targets, Ras und Raf1, erhöhte das Chlamydien-Wachstum und deren Vermehrung. Aufgrund vorheriger in der Literatur beschriebenen Studien wurde bisher angenommen, dass die bei der Chlamydien-Infektion beobachtete Aktivierung der Kinase Erk, die mit der Aktivierung der Phospholipase cPLA2, der Induktion des Interleukins 8 sowie der Stabilisierung des antiapoptotischen Faktors Mcl-1 in Verbindung steht, über den Ras/Raf/Mek-Signalweg vermittelt wird. Ich konnte jedoch zeigen, dass die Chlamydien-induzierte Erk-Aktivierung unabhängig von Ras und Raf1 stattfindet. Vielmehr wird während der Chlamydien-Infektion, Raf1, abhängig von der Kinase Akt, durch Phosphorylierung an Serin259 inaktiviert wird. Zudem wird das inaktivierte Raf1, wiederum abhängig von Akt und dem Adapterprotein 14-3-3β, zur bakteriellen Inklusion rekrutiert. Dies lässt vermuten, dass das Überleben derChlamydien und deren Wachstum nicht nur von der Erk-Aktivierung und dessen Substrate sondern auch von der Inaktivierung von Raf1 und dessen Rekrutierung zur Inklusion abhängt.

Die vorliegende Arbeit liefert erhebliche Beiträge zum tieferen Verständnis zur Rolle von Wirtszellfaktoren bei Chlamydien-Infektionen. Mithilfe einer Funktionsverlust-Hochdurchsatzanalyse konnte ich eine Vielzahl relevanter Gene identifizieren, die insbesondere für die spezifische Regulation von Wirtszellsignalwegen nötig sind, um die Entwicklung der Pathogene und ihr Überleben zu sichern. Die Erkenntnisse dieser Arbeit haben direkten Einfluss auf unser Verständnis von Bakterien-Wirtszellinteraktionen und können dazu beitragen bessere Behandlungsstrategien für Infektionskrankheiten zu entwickeln.

ABSTRACT

Chlamydia trachomatis is a Gram-negative obligate intracellular bacterial pathogen. It is associated with significant human illness, including preventable blindness, reactive arthritis. Furthermore, it is the most common agent of bacterial sexually transmitted diseases with potentially serious sequel in women that include pelvic inflammatory disease, ectopic pregnancy and sterility. As an obligate intracellular pathogen, Chlamydia rely on host cell for all aspects of their survival, from the initial attachment with the host cell membranes, to cellular invasion, acquisition of host cell metabolites and intracellular replication. Unique and largely uncharacterized intracellular host-pathogen interactions are critical for successful chlamydial infection. Despite the prevalence of Chlamydia spp. and their role in human disease, little is known about the mechanisms underlying the infection process, the host pathogen interactions, and the intracellular survival and replication of Chlamydia. To gain a comprehensive understanding of the disease pathogenesis, it is equally important to understand the contribution of the host as well as the pathogen to these complex host pathogen interactions. Therefore in this study I decided to focus on the host factors involved in the Chlamydia infections. For this purpose, silencing of gene expression by RNA interference (RNAi) has proven to be a robust and straight forward technique for gene function analysis in eukaryotic cells. This has become a method of choice for loss of function studies and can serve to unravel the contribution of host cell in the pathogenesis during Chlamydia infection.

This study aims to obtain deeper insights into infection processes of Chlamydia by using RNAi based loss-of-function screen of host cell determinants. As a readout Chlamydia number and inclusion size for the primary infection as well as quantification of Chlamydia progeny (infectivity) were measured to monitor entry, survival and replication of the pathogen within the host. Therefore, a screen was performed in 96 well format, analyzing the impact of ~1500 different human genes mainly covering the human kinome, apoptosis as well as trafficking related genes. With help of various robust statistical analysis methods 134 primary hits were selected for further analysis. To rule out possible off-target effects, which are inherent to the current status of RNAi technology, the primary hits were further validated using 4 new siRNA sequences for each of the identified primary hits. This resulted

in the identification of 59 host cell genes influencing C. trachomatis infection and infectivity. Further analysis of the hits using pathway analysis tools revealed several prominent signaling networks, including Ras/Raf/Mek/Erk pathway. Among the identified targets, knockdown of Ras and Raf1 components of the aforesaid pathway led to increased chlamydial growth and survival. In Chlamydia infections, Erk activation which is believed to be activated through upstream kinases Ras/Raf/Mek is associated with activation of cPLA2, induction of IL8 and stabilization of the anti-apoptotic Bcl-2 family member Mcl-1.

However, I could show that ERK activation after Chlamydia infection is independent of Ras and Raf1. Moreover, it is also evident that in infected cells Raf1 is inactivated by phosphorylation at Ser259 in an Akt dependent manner. Consequently, the Ser259 phosphorylated Raf1 was recruited to the Chlamydia inclusion in an Akt and 14-3-3β dependent manner. This strongly suggests that Chlamydia survival and replication in the host cell depends not only on the activation of ERK and its downstream targets such as cPLA2, but also on the inactivation of Raf1 by phosphorylation and recruitment to the inclusion. The presented work applying RNAi based screening for the dissection of host cell factors involved in C.trachomatis infection gives considerable insights for a broader understanding of how Chlamydia can activate and deactivate specific host cell pathways for their own benefit. The findings of this study have direct implications in our understanding of the Chlamydia disease causing potential and its interactions with the host cells, which might help in evolving better treatment strategies.

1 Introduction

1.1 Chlamydia

Chlamydiales are obligate intracellular bacterial pathogens that can infect a broad range of host organisms. The earliest known descriptions of Chlamydia like diseases, resembling the symptoms of trachoma, are from ancient Chinese and Egyptian writings. However, Halberstädter and von Prowazek were the first to identify the responsible infectious species in 1907, which today is known as Chlamydia trachomatis (Ctr).

Members of the order Chlamydiales are named after the greek word 'chlamys' meaning cloak, based on the incorrect assumption that these organisms are intracellular protozoan pathogens that appear to cloak the nucleus of host cells (Byrne, 2003). Today it is known that these pathogens are Gram-negative bacteria. The observed cloak is in fact the inclusion (Figure 1a) containing numerous individual bacteria.

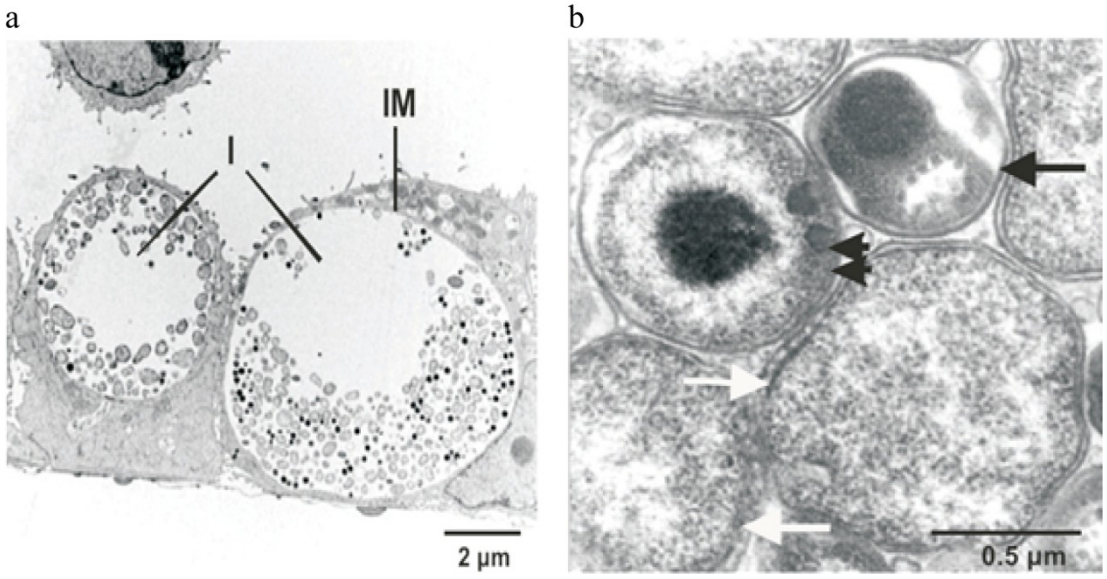


Figure 1: Electron microscopic images of Chlamydia trachomatis (a) Chlamydia trachomatis inclusions 1 day post infection in epithelial cells. (b) Elementary bodies (EBs, black arrow) and reticulate bodies (white arrows). Double arrow heads point to an intermediate body (IB) that started DNA decondensation. I=inclusion; IM=inclusion membrane. (Images kindly provided by Dr. H. Al-Younes, MPI for Infection Biology, Department of Molecular Biology, Berlin.)

Chlamydia spp. have a biphasic developmental cycle, during which they are found in two forms; the metabolically inactive, but infectious extracellular form named elementary bodies (EBs) and the metabolically active, but non-infectious intracellular form, the reticulate bodies (RBs). The development and replication of Chlamydia occur inside host cells in a non-

acidified vacuole termed as inclusion. In order to establish an intracellular niche inside eukaryotic hosts, Chlamydia have evolved or acquired capabilities to enter host cells, replicate inside a suitable environment, evade the immune system, and escape to re-initiate infections in other host cells. Unique and largely uncharacterized intracellular host-pathogen interactions seem to be critical for successful chlamydial infection. As an obligate intracellular pathogen, Chlamydiae rely on host cells for all aspects of their survival, from the initial attachment with the host cell membranes, to cellular invasion, acquisition of host cell metabolites and intracellular replication.

1.1.1 Taxonomy

Until the year 2000 the subdivision of the order Chlamydiales has been mostly based on pheno- and genotypic features (Kaltenboeck, et al., 1993). The only member of this order was the family Chlamydiaceae and two genera. The genus Chlamydia was separated into four different species, C. pecorum, C. pneumoniae, C. psittaci and C. trachomatis. In 2001 a new system of classification has been introduced, based on 16S rRNA and 23S rRNA sequence comparisons (Figure 2). The Chlamydiaceae are now divided based on these rRNA analyses (Bush and Everett, 2001).

The family Chlamydiaceae is divided into two genera, Chlamydia and Chlamydophila. The genus Chlamydia includes the human pathogen C. trachomatis, the mouse pathogen C. muridarum, and the swine pathogen C. suis. The Chlamydophila genus is more diverse with six species, including important human pathogen C. pneumonia and animal pathogens such as C. psittaci, and C. caviae (Pudjiatmoko, et al., 1997; Takahashi, et al., 1997). Based on 16S rRNA sequence relatedness, three new families, Parachlamydiaceae, Simkaniaceae, and Waddliacea (Everett, et al., 1999; Rurangirwa, et al., 1999) were also included into the Chlamydia taxonomy. While these organisms are termed "environmental chlamydiae", some evidence suggests that they may also be associated with clinical disease in humans and animals (Lieberman, et al., 2002).

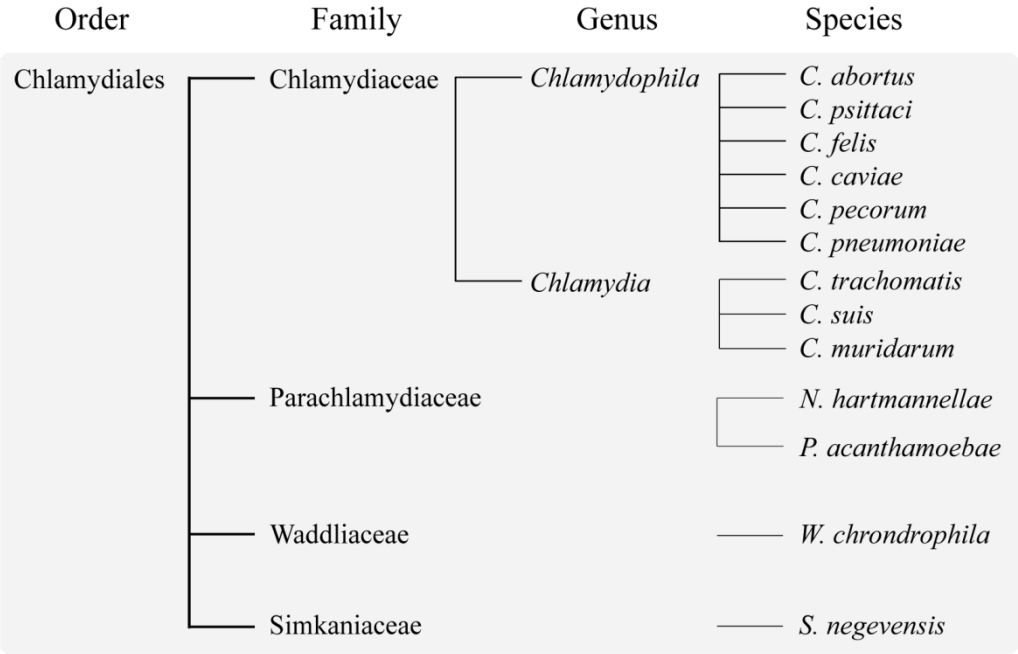


Figure 2: Schematic overview illustrating the new taxonomy of the order Chlamydiales

1.1.2 Diseases caused by human pathogenic Chlamydia strains

Biological differences (biovars) or serological differences (serovars) within C. trachomatis have led to sub classifications into biovars and serovars respectively. C. trachomatis can be separated into the biovars trachoma and the lymphogranuloma (LGV), which is based on clinical, experimental differences and their ability to infect tissue culture cells (Kuo, et al., 1973; Kuo, et al., 1972). Based on serological identification, serovars or serological variants of trachoma and LGV are designated with letters and/or numbers.

The trachoma biovar consist of 14 serovars such as A to K as well as Ba, Da, and Ia. The genital serovars (D, K) are the most common cause of sexually transmitted diseases (Gerbase, et al., 1998), while the ocular serovars (A, B, Ba or C) cause the blinding trachoma (Schachter, 1978). Infections by strains of the trachoma biovar are confined to mucosal epithelia, such as the urogenital tract or the conjunctiva (Schachter, 1978). They are associated with trachoma, an infection of conjunctival epithelia that, through persistence and re-infection, causes a chronic disease that leads to blindness. In developing countries, trachoma has an estimated annual incidence of 80 million cases with 6 million individuals blinded as a result of this disease (Thylefors, et al., 1995). An estimated 92 million adults

worldwide contract sexually transmitted chlamydial infection each year and are associated with 60% of tubal infertility cases and 40% of ectopic pregnancy cases (Peipert, 2003). Sexually transmitted C. trachomatis infection results in a variety of anomalies including mucopurulent cervicitis, urethritis, salpingitis, lymphogranuloma venereum, endometritis, opthalmia neonatorum, and infant pneumonia (Schachter, 1978).

The LGV biovar consists of four serovars, L1, L2, L2a and L3, which can invade lymphatic tissue. LGV biovar infections originate at a mucosal site, but serious disease is associated with systemic dissemination and proliferation in cells of the lymph nodes (Schachter and Osoba, 1983). A recent work suggests that, previous infection of C. trachomatis increases the likelihood of HIV and/or HPV transmission, demonstrating that the chlamydial infections increase the risk of HIV transmission at least three- to five-fold (Nusbaum, et al., 2004).

C. pneumoniae species were described as respiratory pathogens infecting many humans in all areas of the world (Kuo, et al., 1995). Epidemiological studies of C. pneumoniae have shown that the pathogen is very common worldwide with 60-70% of adults being seropositive. Transmission appears to be from person to person. The pathogen is a cause of a broad spectrum of respiratory tract infections with a tendency to become chronic and reinfection is a common event. The most serious outcome of respiratory tract infection is pneumonia. Clinical manifestations include a wide spectrum of diseases such as bronchitis, pneumonitis, sinusitis, and acute pharyngitis. Cases of primary infection are more severe and prolonged than those of reinfection. There is evidence that C. pneumoniae is responsible for atherosclerotic cardiovascular disease, asthma and sarcoidosis (Cochrane, et al., 2005; Cuffini, et al., 2006; Kalayoglu, et al., 2002).

C. psittaci is widely distributed and affects many mammalian and avian species causing genital, intestinal, conjunctival, or respiratory infections. Genital infections with C. psittaci have been well characterized and can cause abortion and infertility. Although mammalian strains of C. psittaci are not known to infect humans, avian strains occasionally are known to cause infection in humans leading to pneumonia and the systemic illness known as psittacosis (Harkinezhad, et al., 2009). In general, chlamydial infection causes mild diseases with persistent infections and poor immunity.

1.1.3 The developmental cycle of Chlamydia

The Chlamydia developmental cycle, as know today was first described by Bedson and Bland in 1932. It consists of two distinct morphological and functional forms, the elementary body (EB) and the reticulate body (RB). The developmental cycle occurs within a membrane-bound vacuole, called "inclusion". The life cycle of Chlamydiae is a transition between EB into RB, RB into RB, and RB into EB (Figure 3**Error! Reference source not found.**).

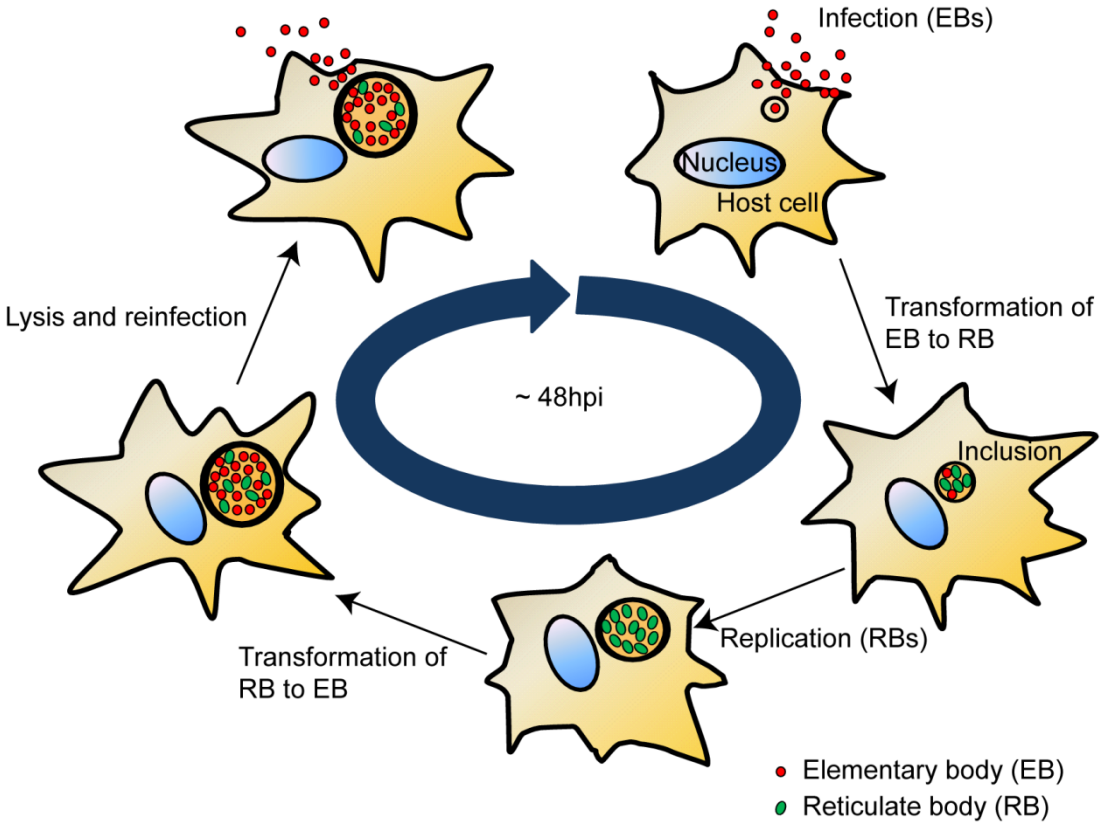


Figure 3: Schematic overview of *Chlamydia* life cycle. *Chlamydiae* have two developmental forms. The elementary body (EB), shown as red circles is infectious, metabolically inactive and small (0.2-0.3 μm). The reticulate body (RB) shown as green circles is larger (1.0 μm), non-infectious and metabolically active. The whole cycle takes between 24 – 72 h depending on species.

The elementary body (EB) is a small (0.3 μm), round, electron-dense, 'spore-like' metabolically inert but infectious form of the organism, whereas RB is larger (1 μm) then EB, have less condensed structure, non-infectious and metabolically active (Matsumoto, 1973).

1.1.3.1 Attachment and entry

Chlamydiae are capable of attaching to and infecting non-phagocytic cells from various animal species. The attachment depends on polysaccharides and heparin sulphate (Spears

and Storz, 1979; Wuppermann, et al., 2001; Zhang and Stephens, 1992) by establishing strong bonds between host cells and bacteria (Duensing, et al., 1999).

Chlamydiae have evolved extremely efficient means of invading nonphagocytic cells. For this, more specific interactions with so far unidentified host-receptors are thought to be involved (Dautry-Varsat, et al., 2005). After cell adhesion, the Chlamydiae are either endocytosed or phagocytosed, both clathrin-dependent and independent mechanisms are proposed (Dautry-Varsat, et al., 2005). Immediately after irreversible binding, type III secretion system (TTSS) exports Tarp protein (Translocated actin-recruiting phosphoprotein, CT456) into the host cell (Clifton, et al., 2004). Where it is phosphorylated at tyrosine residues, this leads to remodelling of the cytoskeleton (Clifton, et al., 2004). All these findings show that chlamydial EBs contain functional TTSS that is capable of delivering signals inside the host cell prior to differentiation into RB. Assembly of TTSS into functional complexes and expression of early effector molecules such as Tarp may be accomplished during redifferentiation of RB into EB. This may furnish metabolically inert EBs with molecules that are active upon EB attachment and important for invasion into the host cell. The key question which still remains elusive is the identification of chlamydial ligands responsible for the attachment to host cells. Several chlamydial proteins are exposed at the surface and that makes them potential candidates for the role of adhesin proteins. Among these proteins are OmcB, OmpA and HSP70 (MOMP). Pmp proteins are also suggested to be involved in attachment (Everett and Hatch, 1995; Grimwood, et al., 2001; Grimwood and Stephens, 1999). Several important signal transduction pathways are also known to be involved during the entry of Chlamydia in to the host cells, such as small GTPases (Rho, Rac, Cdc42, Arf6), PI3- kinases or MEK/ERK (Dautry-Varsat, et al., 2005) leading to host cytoskeleton rearrangements, thus facilitating the pathogen entry.

1.1.3.2 Intracellular development

For any intracellular pathogen the development inside a host cell is a significant challenge. However, for this purpose intracellular parasites have evolved various strategies to escape lysosomal killing. One of the key routes for surviving is by occupying distinct compartments within the host cell. Pathogens like Shigella, Listeria or Rickettsia replicate in the cytoplasm (Hackstadt, 1996; Hackstadt, 1998; Marquis, et al., 1997). Others like Leishmania or Coxiella

even survive harsh acidic conditions inside the lysosome, which are even supportive for their own metabolism (Hackstadt and Williams, 1981; Mukkada, et al., 1985). Chlamydia is a member of another group of intracellular parasites that reside in a unique vesicle which does not fuse with lysosomes. For most cases, the biogenesis of these vacuoles is not well understood. This strategy of forming non- lysosomal vacuoles for surviving inside host organisms is another survival technique used by many intracellular parasites such as the bacteria M. tuberculosis, M. avium, L. pneumophila, S. typhimurium and Chlamydia as well as the protozoan Toxoplasma gondii (Garcia-del Portillo and Finlay, 1995; Rikihisa, 1991; Wells and Rikihisa, 1988).

Another specific feature of the chlamydial inclusion is the lack of acidification which would prevent the optimal activity of acid hydrolases. It was demonstrated that, in cells that phagocytose both yeast and Chlamydiae, the vacuoles containing Chlamydia are protected from phagolysosomal fusion (Eissenberg and Wyrick, 1981). It was also revealed that early inclusions with EB are prevented from maturation into phagolysosomes (Eissenberg, et al., 1983) and inhibition of chlamydial protein synthesis leads to fusion with lysosomes (Scidmore, et al., 1996).

During the first four hours after Chlamydia entry into the host cells many events occur. At first, the EB endosome pH drops to 6.2 and then stabilizes at 6.6, the initial drop in pH may be required for the recycling of the receptor(s) to the cell surface in addition might play a role in catalyzing the conversion of metabolically inactive EB to metabolically active RB and the later stabilization of the pH at 6.6 might help Chlamydia to avoid lysosomal fusion (Schramm, et al., 1996). The endosome containing EB escapes fusion with lysosomes, within two hours after infection the EB-containing vesicle is devoid of markers that distinguish early and late endosomes or lysosomes. Phosphorylation of epithelial proteins triggered by the EB attachment results in rearrangement of the host cell cytoskeleton (Birkelund, et al., 1994; Fawaz, et al., 1997). Local accumulation of F-actin and clathrin helps to redistribute endosome containing EB to the perinuclear region (Majeed and Kihlstrom, 1991). This translocation depends on dynein motor driven movement of the EB vacuoles on microtubules (Clausen, et al., 1997; Schramm and Wyrick, 1995). It was also discovered that, if the intracellular concentration of calcium remains at homeostatic level, EB-containing endosomes can fuse with one another, but not with lysosomes (Majeed, et al., 1994). Early

Chlamydia gene expression leads to vacuole modification and subversion of the EB trafficking from the endocytic to the exocytic pathway.

Approximately six hours after internalization, the transition of EBs into RBs is complete and metabolically active RBs enter the logarithmic growth stage, with a generation time of 2-2.5 hours, which continues until 24-40 hours after infection. Very little is known about the function and composition of the inclusion membrane that forms the Chlamydia inclusion. As bacteria grow and proceed through their developmental cycle, the inclusion also grows and expands. This process is not dependent on host protein synthesis because the chlamydial inclusion develops normally in cells treated with the inhibitor cycloheximide. The inclusion can intercept vesicles released from the Golgi apparatus thereby capturing mostly sphingolipids, phospholipids and cholesterol (Hackstadt, et al., 1997; Scidmore, et al., 1996). Activation of cPLA2 through Mek/Erk signaling pathway was shown to be essential for chlamydial acquisition of host glycerophospholipids (Su, et al., 2004). Recently it was also shown that Chlamydia induces fragmentation of Golgi apparatus to form ministacks for efficient acquisition of lipids (Heuer, et al., 2009). Other nutrients such as amino acids (Hatch, 1975; Karayiannis and Hobson, 1981), Nucleotides (McClarty and Qin, 1993) or iron (Al-Younes, et al., 2001) need to be recruited from the host cell as well.

In addition to the re-routing of endosomal traffic, intracellular Chlamydiae also disturb other cellular functions: N-cadherin dependent cell-cell junctions are disrupted and apoptosis is inhibited (Greene, et al., 2004; Rajalingam, et al., 2001). The MHC Class I and Class II response is also disturbed through the secretion of a protease which cleaves transcription factors of MHC antigens (Heuer, et al., 2003; Zhong, et al., 2001).

1.1.3.3 Persistence

As an interruption of the classic lifecycle, persistent infections can occur, which are induced by external stimuli. During persistence, RBs do not redifferentiate into EBs, but develop into so called aberrant bodies (Abs) with significantly modified morphology (de la Maza, et al., 1987). The bacteria stay metabolically active but are unable to establish a productive infection. By this means, Chlamydiae are inaccessible to the host's immune system and can survive for a long period of time. If the stimulus is removed, the bacteria are reactivated and continue to undergo the regular, acute developmental cycle.

1.1.3.4 Exit from the host cell

Escape into the environment is the final step of the chlamydial life cycle. Despite the apparent simplicity of the exiting process, the real mechanisms that direct the end of the developmental cycle and trigger transition of RB back to infectious EB are almost completely unknown. This process may involve multiple events. A decrease in host nutrients may signal detachment of RB from the inclusion membrane and activation of histone proteins. Histone proteins can regulate stage-specific expression of genes and initiate DNA condensation (Barry, et al., 1992; Tao, et al., 1991).

The fact that different species of Chlamydiae exit host cells in different ways supports active and complex processes behind this final stage of the developmental cycle. Some Chlamydiae lyse the host cell when exiting, whereas others exit in a less aggressive manner. For example, C. trachomatis serovar D (UW3 isolate) exit cells by exocytosis. The inclusion moves along the exocytic pathway to the epithelial surface for fusion with the plasma membrane. During this process the inclusion exvaginates at the plasma membrane and the host cell remains viable (Todd and Caldwell, 1985). Other serovars of C. trachomatis, such as LGV strains, destroy the host cell when exiting. It was reported that Chlamydiae encode a cytotoxin with a unknown role (Belland, et al., 2001). It is possible that this toxin is involved in the process of releasing bacteria from the cell.

1.2 RNA interference

A turning point for large-scale functional studies in animals was the discovery that long double-stranded (ds)RNA triggers potent and specific mRNA degradation in C. elegans through the evolutionarily conserved RNA interference (RNAi) process (Figure 4) (Fire, et al., 1998).

Because long dsRNA can be easily and reproducibly synthesized and introduced into C. elegans RNAi became soon a standard method and expanded rapidly from single-gene to full genome functional studies in this model organism (Fraser, et al., 2000; Gonczy, et al., 2000; Kamath, et al., 2003). While this approach has been successful in model organisms such as D. melanogaster (Kennerdell and Carthew, 1998), the use of long dsRNAs for RNAi in most

mammalian cells was hampered by the induction of a strong, non-specific interferon response by dsRNAs longer than 30 bp (Stark, et al., 1998).

The analysis of the molecular mechanism of RNAi revealed that long dsRNA is cleaved by the RNase III-like enzyme Dicer into 21-23 nt short interfering (si)RNAs, which are the ultimate mediators of RNAi (Elbashir, et al., 2001). Further analyses revealed that siRNAs are targeted to the RNA induced silencing complex (RISC) (Martinez, et al., 2002), where they are unwound by the Argonaute2 protein (Meister, et al., 2004). If the antisense strand is loaded into RISC it triggers degradation of (partially) complementary mRNA. Thus, siRNAs elicit mRNA degradation without inducing the interferon pathway and have therefore enabled the use of RNAi as a functional genomic tool in mammals.

For mammalian RNAi experiments a range of technologies that closely mimics the endogenous Dicer cleavage products has been developed. Today, the most commonly used approaches are chemically synthesized siRNAs (Elbashir, et al., 2001), which were historically the first RNAi reagents introduced for mammalian tissue culture cells and vector-expressed short hairpin (sh)RNAs that are converted intracellularly into siRNAs (Brummelkamp, et al., 2002).

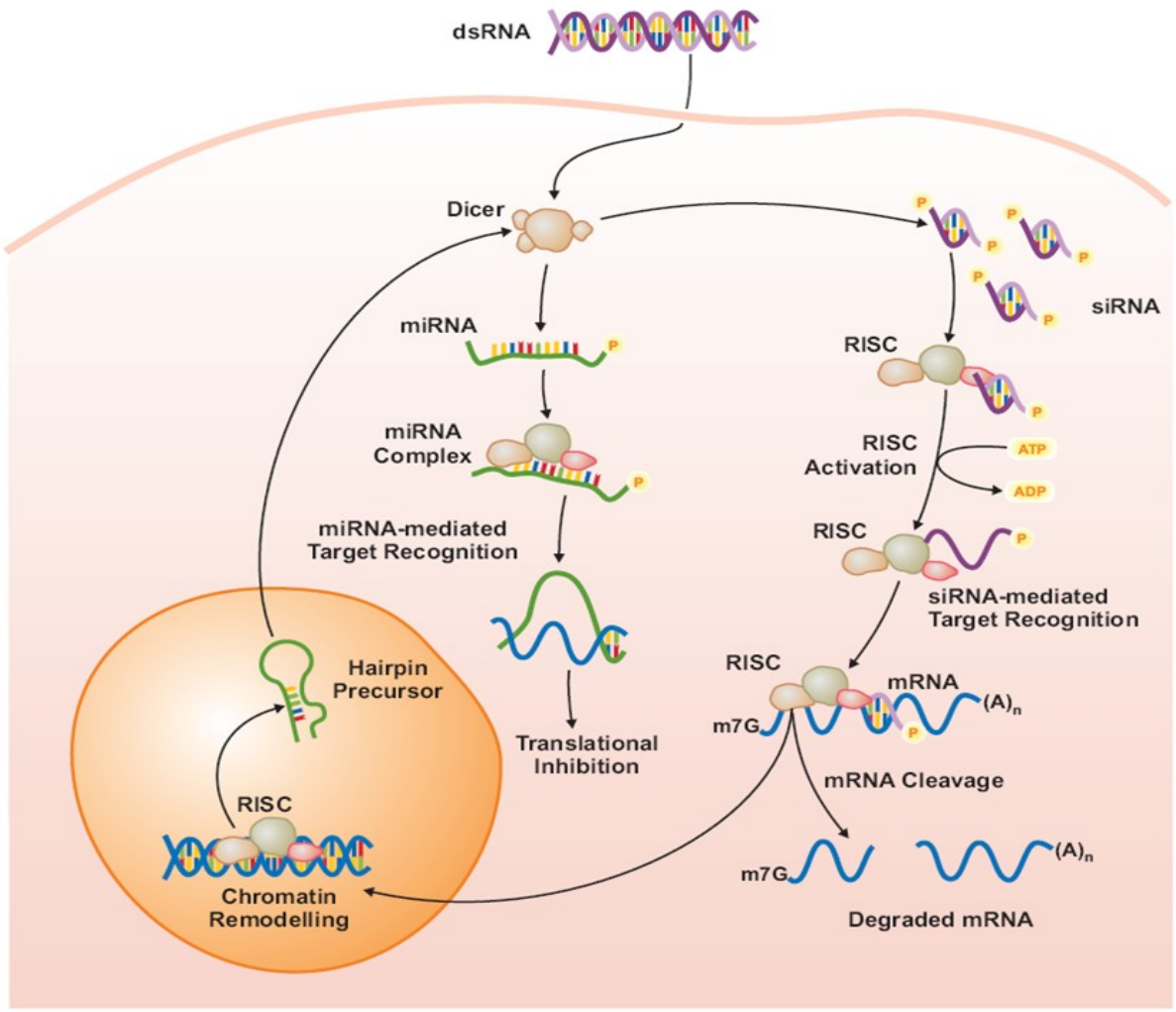


Figure 4: Overview of mechanism of RNA interference

Several large-scale libraries for the human and mouse genomes have been recently generated with these two technologies and have been made available through a number of suppliers (Echeverri and Perrimon, 2006). Both, siRNA and shRNA libraries require the synthesis of either RNA or DNA oligonucleotides that are transfected directly or first cloned into an expression vector, respectively.

Both approaches have advantages and disadvantages depending on the experimental context and the price aspect (Echeverri and Perrimon, 2006). An important requirement for any large-scale RNAi study is a high knockdown efficiency for each individual silencing trigger in a library. For this purpose, the most recently released siRNA and shRNA libraries have used design algorithms that are based on thermodynamic and sequence-specific properties to predict efficient silencing molecules (Boese, et al., 2005; Silva, et al., 2005). While the use

of these algorithms has improved the overall silencing efficacy, no prediction algorithm exists yet that can exclude off-target gene silencing, which has been recently recognized as a major challenge for the reliability of data generated in screen using siRNAs or shRNAs (Jackson, et al., 2003; Lin, et al., 2005). Detailed analyses revealed that siRNAs and shRNAs in addition to their specific targets down regulate numerous unintended transcripts with limited sequence complementarity to the specific siRNA target sequence (Birmingham, et al., 2006; Jackson, et al., 2006), which makes the design of fully specific silencing molecules difficult if not impossible.

1.2.1 Functional RNAi screening applied to study host pathogen interactions

The use of RNA interference (RNAi) to rapidly and efficiently inhibit the expression of proteins (Bass, 2000) offers the possibility of carrying out unbiased reverse genetic screens to identify host proteins critical in microbial pathogenesis. Several screens have already shed light on various cellular processes such as cell viability (Boutros, et al., 2004), cytokinesis (Eggert, et al., 2004), wnt Signaling (DasGupta, et al., 2005), JAK/STAT Signaling (Baeg, et al., 2005), and mechanisms of host–pathogen interaction, including Listeria and Mycobacterium pathogenesis (Agaisse, et al., 2005; Cheng, et al., 2005; Philips, et al., 2005), Candida albicans phagocytosis (Stroschein-Stevenson, et al., 2006), L. pneumophila exploitation of the early secretory pathway (Dorer, et al., 2006) and involvement of PDGFR, Abl kinases (Elwell, et al., 2008) as well as Tom complex (Derre, et al., 2007) for C. trachomatis infection. Most of the above mentioned screens were done using the D. melanogaster cells.
In this study we established and performed an RNAi based screen in human cells for the first time to study C. trachomatis and host cell interactions.

1.3 Ras/Raf/Mek/Erk Signaling cascade

The Ras/Raf/MEK/ERK (Figure 5**Error! Reference source not found.**) signaling was the first MAP kinase cascade to be characterized. It is probably one of the most well known signal transduction pathways among biologists because of its implication in a wide variety of cellular functions as diverse -and occasionally contradictory- as cell proliferation, cell-cycle arrest, terminal differentiation and apoptosis. The initial step of the Raf/MEK/ERK cascade is

the activation of Raf1 by direct interaction with a GTP-Ras protein (McCubrey, et al., 2007). The GTP-bound active Ras can recruit Raf1 to the cell membrane by binding to the Ras-binding domain of Raf1 (C-Raf). Once at the cell membrane Raf1 can be activated by multiple phosphorylations on serine, threonine, and tyrosine residues (Morrison and Cutler, 1997). Even though the exact mechanism of Raf1 activation is still a matter of controversy it can be summarized in a following way based on the existing knowledge. Amino-terminal part of Raf1 is shown to be involved in negative regulation of its enzymatic activity as its truncation results in constitutive activation of the kinase activity (Morrison and Cutler, 1997). Phosphorylation of Tyr340 and Tyr341 sites are important for Raf1 activation by both receptor and non-receptor tyrosine kinases (Marais, et al., 1995; Stokoe and McCormick, 1997). Also Ser338 and Ser339 of Raf1 can be phosphorylated, and mutation of these residues partially blocks activation of Raf1 (Diaz, et al., 1997).In contrast to this, phosphorylation of Raf1 at Ser259 has an inhibitory effect. When phosphorylated, Ser259 of Raf1 becomes a binding site for the 14-3-3 protein (Muslin, et al., 1996), a negative regulator of Raf1. This residue may play a negative regulatory role, because its mutation to alanine results in an active kinase (Rommel, et al., 1997). So far exact mechanism through which 14-3-3 binding inhibits Raf1 is ambiguous. While some models suggest that 14-3-3 binding may hold Raf1 in an inactive conformation, the others suggest that 14-3-3 could mediate interaction between Raf1 and a tyrosine phosphatase that dephosphorylates Tyr340 and Tyr341 tyrosines (Xia, et al., 1999). Raf1 also contains a second 14-3-3 binding site at Ser621, which has been shown to have a positive regulatory effect on Raf1 signaling (Ferrier, et al., 1997). Taken together, 14-3-3 appears to have both positive and negative effects on Raf1 signaling, depending on cell type and assay conditions (Morrison and Cutler, 1997).

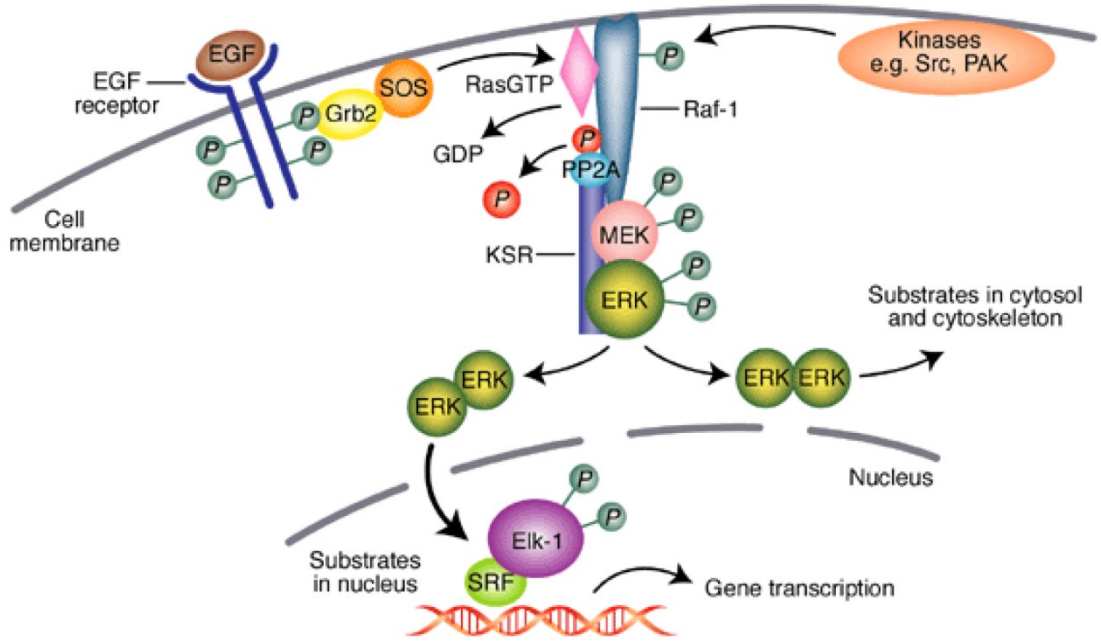


Figure 5: Overview of Ras/Raf/MEK/ERK pathway. This figure illustrates how the Raf/MEK/ERK pathway is regulated by Ras as well as various kinases, which serve to phosphorylate S/T and Y residues on Raf. Some of these phosphorylation events serve to enhance Raf activity, whereas others serve to inhibit Raf activity Moreover, there are phosphatases such as PP2A, which remove phosphates on certain regulatory residues. Activated ERK can enter the nucleus and phosphorylate transcription factors or activate its substrates in the cytosol. Adopted from (Kolch, et al., 2002).

Upon activation by kinases (e.g PAK, Src family kinases, and potentially PKC) Raf1 then phosphorylates mitogen-activated protein kinase (MEK) 1 and 2, which are dual specificity protein kinases that target tyrosine and serine/threonine residues for phosphorylation. All three Raf family members are able to phosphorylate and activate MEK with different biochemical potencies (Alessi, et al., 1994). (Figure 5) The predominant downstream target of MEK1/MEK2 is ERK (McCubrey, et al., 2007) a serine/threonine kinase existing in two isoforms ERK1 and ERK 2. The activities of ERK1 and ERK2 are positively regulated by phosphorylation of both T202 and T204 in the activation loop mediated by MEK1 and MEK2 (McCubrey, et al., 2006). ERK can directly phosphorylate many transcription factors including Ets-1, c-Jun, c-Myc, S6 kinase (p90Rsk), and NFkB (Chang, et al., 2003; Nakano, et al., 1998) and is also known to phosphorylate cPLA2 (Hiller and Sundler, 1999).

There are various studies demonstrating that the Chlamydia infection leads to activation of Erk followed by the down-stream activation of cPLA2 (Su, et al., 2004), an induction of IL8 (Buchholz and Stephens, 2008), TnfR1 shedding (Paland, et al., 2008) and stabilization of Mcl-1 (Rajalingam, et al., 2008).

2 Aim of the study

Chlamydia trachomatis is a Gram-negative obligate intracellular bacterial pathogen and is the cause of significant human illness, including preventable blindness, reactive arthritis. Furthermore it is the most common agent of bacterial sexually transmitted diseases with potentially serious sequel in women that include pelvic inflammatory disease, ectopic pregnancy and sterility. Chlamydia spp. have a biphasic developmental cycle, during which they are found in two forms; the metabolically inactive, but infectious extracellular form named elementary bodies (EBs) and the metabolically active, but non-infectious intracellular form, the reticulate bodies (RBs). The development and replication of Chlamydia occur inside host cells in a non-acidified vacuole termed as inclusion. In order to establish an intracellular niche inside eukaryotic hosts, Chlamydia have evolved or acquired capabilities to enter host cells, replicate inside a suitable environment, evade the immune system, and escape to re-initiate infections in other host cells. Unique and largely uncharacterized intracellular host-pathogen interactions seem to be critical for successful chlamydial infection. As an obligate intracellular pathogen, Chlamydiae rely on host cells for all aspects of their survival, from the initial attachment with the host cell membranes, to cellular invasion, acquisition of host cell metabolites and intracellular replication.

Despite the prevalence of Chlamydia spp. and their role in human disease, little is known about the mechanisms underlying the infection process, the host pathogen interactions, and the intracellular survival and replication of Chlamydia. In spite of intense research genetic manipulation of Chlamydiae still remains impossible. Even though several options for generating a universal genetic system are currently being pursued, the anticipated success of these approaches is uncertain. On the other hand, silencing of gene expression by RNA interference (RNAi) has proven to be a robust and straight forward technique for gene function analysis in eukaryotic cells and has become a method of choice for loss of function studies. This affords the opportunity to uncover the novel host-pathogen interactions in cellular systems. In the past few years several RNA interference screens have been performed to study of host pathogen interactions including Listeria and Mycobacterium pathogenesis, Candida albicans phagocytosis as well as the L. pneumophila exploitation of the early secretory pathway.

In order to obtain a comprehensive insight in infection processes of Chlamydia, we decided to perform a loss-of-function screen of host cell determinants using high-throughput RNA interference. As readout we chose Chlamydia number and inclusion size for the primary infection, which would give us insights in to the pathogens entry and Chlamydia infective progeny (infectivity) to measure the survival and replication of the pathogen within the host. Therefore, the goal was to perform a screen in 96 well format, analyzing the impact of ~1500 different human genes mainly covering the human kinome, apoptosis as well as trafficking related genes. Through this approach we aimed to identify host cell determinants, which influence Chlamydia entry and survival and replication with in host cells. The targets thus obtained were to be used for extensive functional analysis to determine the molecular mechanisms underlying the observed phenotypes and to identify the host cell processes exploited by Chlamydia.

3 Results

3.1 Assay development and optimization of parameters

Performing a successful high throughput RNAi screen needs a robust and specific assay. Developing an assay requires careful attention to every minor parameter. Also consideration has to be paid for establishing all the parameters with a thought for possible variations that can have a direct or indirect influence on the assay results. The assay specifically designed for studying Chlamydia infection and infectivity involve multiple parameters with several liquid handling steps. Therefore, all parameters of the assay should work synergistically introducing least possible variations due to handling. The following section of the results gives an overview of the assay and describes the optimization of each parameter in the development of this assay.

3.1.1 Overview of the Chlamydia Infectivity Assay

The general scheme of the assay which was developed hitherto is shown in Figure 6**Error! Reference source not found.**. The assay was aimed to identify host cell factors relevant for Chlamydia trachomatis infection and completion of the life cycle. For this we have set up a tripartite functional assay (Figure 6). At day 1 HeLa cells were transfected with the same siRNAs on three plates. The first plate was fixed 3 days post transfection to monitor the effect of the gene knockdown on the cell number. At the same day, the two other plates were infected with C. trachomatis, one of which was used to analyze the effect of gene knockdown (KD) on the primary infection. The third plate was lysed 48 hours post infection (hpi) and dilutions were used to infect non-transfected HeLa cells to identify genes required for the completion of C. trachomatis life cycle. After fixation, cells were stained with Hoechst dye for counting cell numbers and with an antibody directed against the major outer membrane protein (MOMP) of C. trachomatis. Nuclei and Chlamydia inclusions were acquired by automated microscopy and images were analyzed using an automated image analysis software (Scan^R, Olympus). As the main parameters, the number of inclusions per cell, and the inclusion size have been determined.

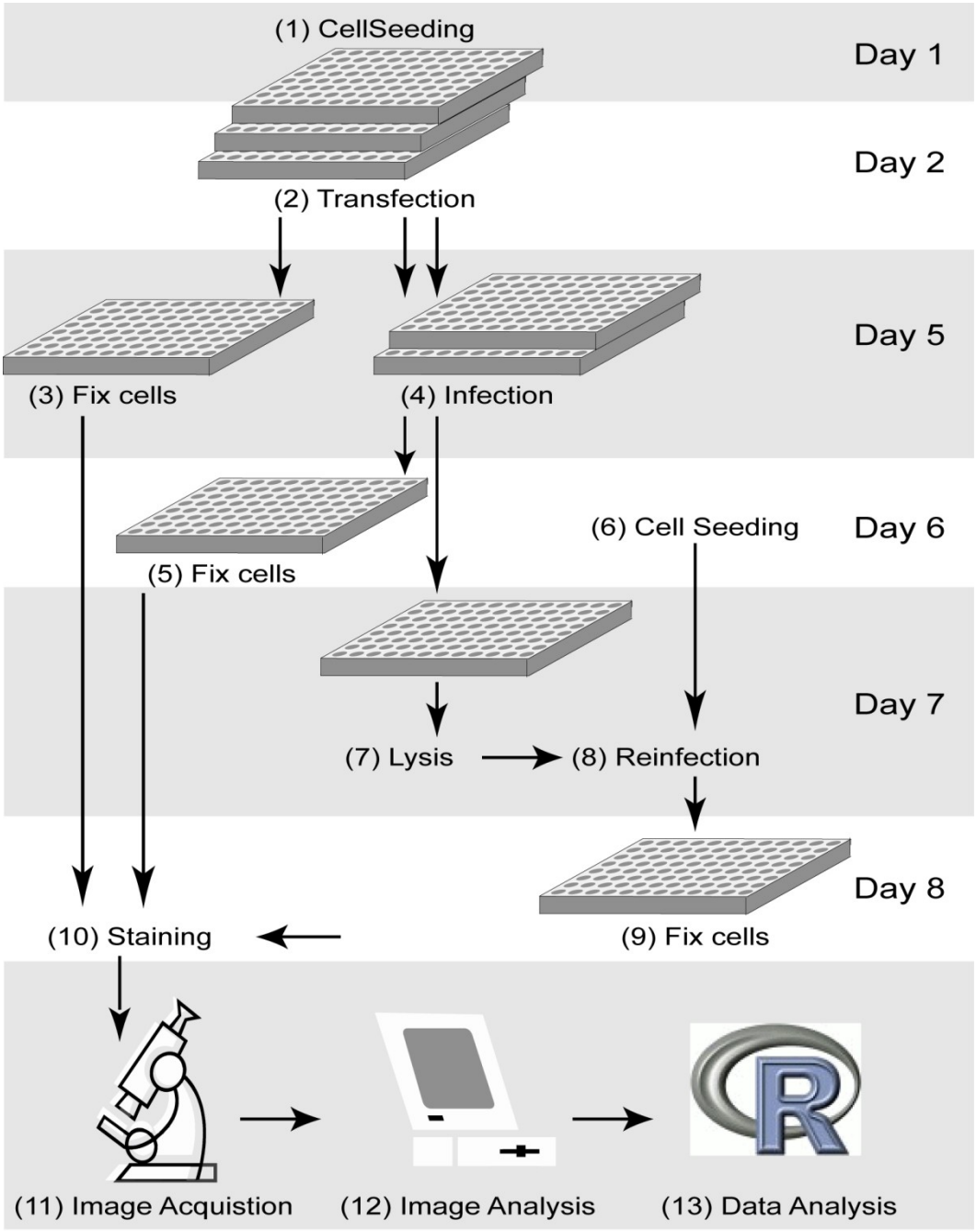


Figure 6: Infectivity assay overview. HeLa cells were seeded (1) and transfected (2) in triplicate plates. 72h post transfection (pt) one plate was fixed (3) to control for siRNA specific effects on cell growth. The remaining two plates were infected with Ctr (4) and 24h post infection (pi) one of the infected plates was fixed to evaluate the number and size of Ctr infectious particles (infection) (5). Fresh cells were seeded (6) and infected with the lysate from the remaining infected plate 48h post infection (7+8). The reinfected plate was fixed 24h post reinfection (9) for evaluation of the Ctr infectious progeny (infectivity). All plates were stained for host nuclei and with a Ctr specific antibody (10), images were acquired using automated microscopy (11), analyzed and the data exported for further analysis (12+13).

3.1.2 Image acquisition and analysis

An important step in large scale screening is the automation image acquisition and analysis. Since such a large scale screen produces thousands of images to be analyzed to extract various parameters, based on which the hits will be identified. Therefore, it is imperative to automate this critical step of the screen. We used Scan^R automated microscopy system from Olympus for the acquisition of images. The screening plates generated were stained with Hoechst dye for counting cell numbers and with an antibody directed against the major outer membrane protein of C. trachomatis using the staining protocol described in 5.2.6. Throughout the screen 4 images/well were acquired for host nuclei in Hoechst channel and for Chlamydia inclusions in Cy3 channel shown in blue and red respectively in Figure 7**Error! Reference source not found.**. Subsequently we developed a custom analysis method using the Scan^R analysis software from Olympus. Analysis was based on gating the populations using the fluorescence, size, form and intensity parameters in the respective channels for nuclei and Chlamydia inclusions. The data extracted for various parameters such as number of objects, size and intensities etc were then exported for further analysis.

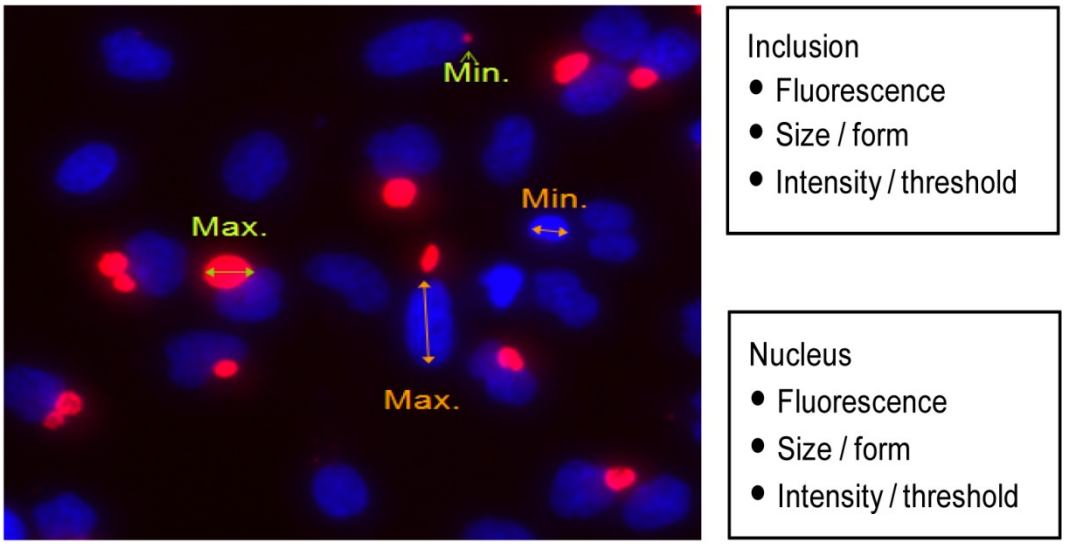


Figure 7: Overview of the image analysis with Scan R analysis software. Example of the image analysis with the different parameters marked on the microscopic image, shown in red (Chlamydia inclusions) and blue (Nuclei). The parameters used for the gating, classification of both Chlamydia inclusions and nuclei are shown in the right panel.

3.1.3 Optimizing Chlamydia infection conditions

To establish Chlamydia infection in 96 well plates and also to adopt an optimal infection procedure, following infection conditions were tested. Volume of the infection medium,

washing the cells with phosphate buffered saline after 2 hpi before replacing with fresh medium, replacing the medium without washing 2 hpi, no medium replacement as well as shaking and no shaking of the plates during the first 2 hours of infection (Figure 8). For this HeLa cells seeded at a density of 8000 cells/well 1 day before were infected with an MOI 0.5 of C. trachomatis. Elementary body's were resuspended either in 100 µl or 50 µl of infection medium and added to the cells in 96 well plates after removing the medium. The plates were subjected to shaking every 30 minutes or left without during first 2 hours of infection.

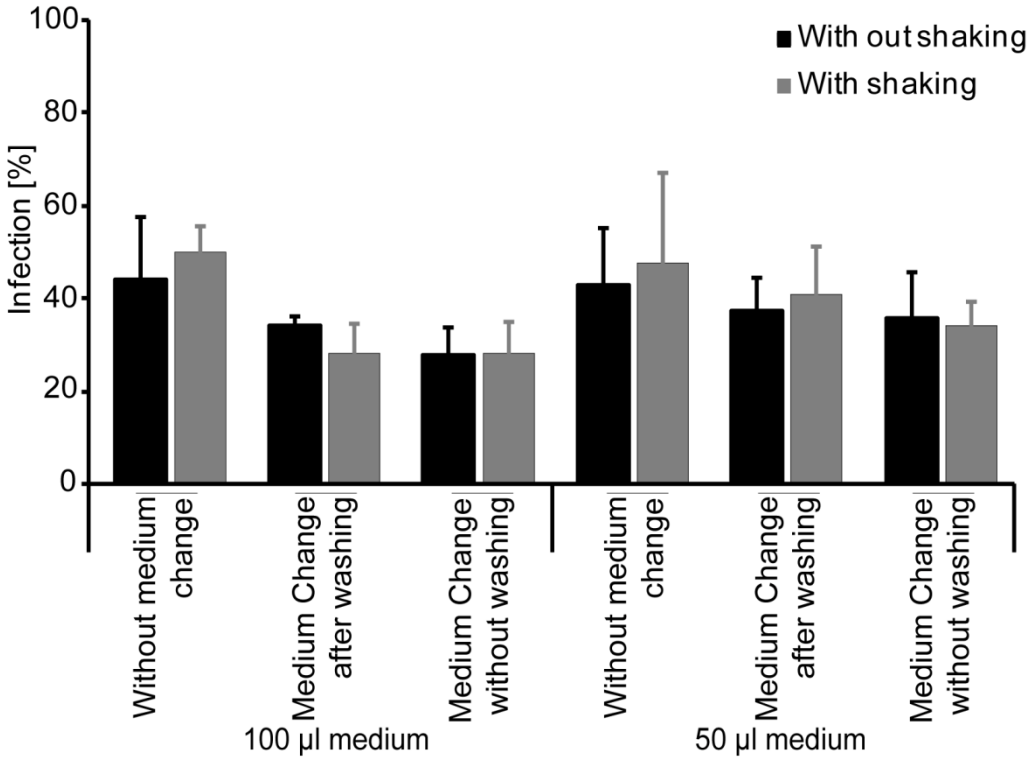


Figure 8: Optimizing Infection of C. trachomatis in 96 well plates. C. trachomatis resuspended in either 100 µl or 50 µl of infection medium were used to infect HeLa cells at an MOI of 0.5 in 96 well plates. During the first 2 hours of infection plates were subjected to shaking (gray bars) or left without (black bars) every 30 minutes. Additional infection conditions were tested as indicated in the figure. Images were acquired and analyzed to determine % infection. The results represent means ± SD from three independent experiments.

Additionally after the first 2 h of infection cells were treated differently, i.e. Chlamydia were left on the cells without medium replacement, cells were washed with PBS once and 100 µl of fresh infection medium added for the rest of the infection duration, or medium was replaced with 100 µl of fresh infection medium without washing the cells 2 h post infection. 24 h later cells were fixed and were stained with Hoechst dye for counting cell numbers and with an antibody directed against the major outer membrane protein (MOMP) of C.

trachomatis. Nuclei and Chlamydia inclusions were acquired by automated microscopy and images were analyzed using the Scan^R software and % infection was calculated.

It was found that shaking the plates and the volumes of the infection medium used for resuspending the EB's did not have any influence on the rate of infection. Conversely leaving Chlamydia on the cells without medium replacement 2 h post infection resulted in slightly higher infection rate which could be attributed to development of secondary inclusions as a result of asynchronous infection, which we wanted to avoid for the screen to keep the conditions uniform. Also between the conditions of washing the cells or just a medium replacement without washing no difference could be detected, although infection with 50 µl infection medium resulted in slightly better infection rate. Finally I decided to use the condition where cells were infected with EB's resuspended in 50 µl of infection medium, which was replaced with 100 µl of fresh infection medium 2 h post infection without washing the cells.

3.1.4 Optimizing detergent concentration for cell lysis

It was necessary to design a method for cell lysis, which would lead to complete lysis of the C. trachomatis infected cells and does not have an effect on the target cells on to which the supernatant is passaged. As the screen was intended to be performed in 96 well plates, it was not possible to use the conventional method of cell lysis using glass beads commonly used by the Chlamydia researchers. Therefore I tested two different detergents a non-ionic Nonidet P40 and a zwitterionic detergent CHAPS at final concentrations ranging from 0.01 to 0.1% (Figure 9**Error! Reference source not found.**). HeLa cells plated at a density of 8000 cells/well were infected with C. trachomatis at an MOI 0.5 in 96 well plates. 48 hpi cells were lysed with 0.1, 0.06, 0.03 and 0.01% final concentrations of either Nonidet P40 or Chaps detergents, serial dilutions of the lysates were used to infect cells seeded one day before. 24 h later cells were fixed were stained with Hoechst dye for counting cell numbers and with an antibody directed against the MOMP protein of C. trachomatis. Nuclei and Chlamydia inclusions were acquired by automated microscopy and images were analyzed using the Scan^R software and the % infection was calculated. Nonidet P40 at a final concentration of 0.06% was found to be most effective in complete lysis of the cells. It also did not affect the cell viability of the target cells in the infectivity plates. This was determined by counting the

cell numbers of the target cells of the infectivity plate. Therefore this concentration of the detergent was chosen for further experiments and the screen.

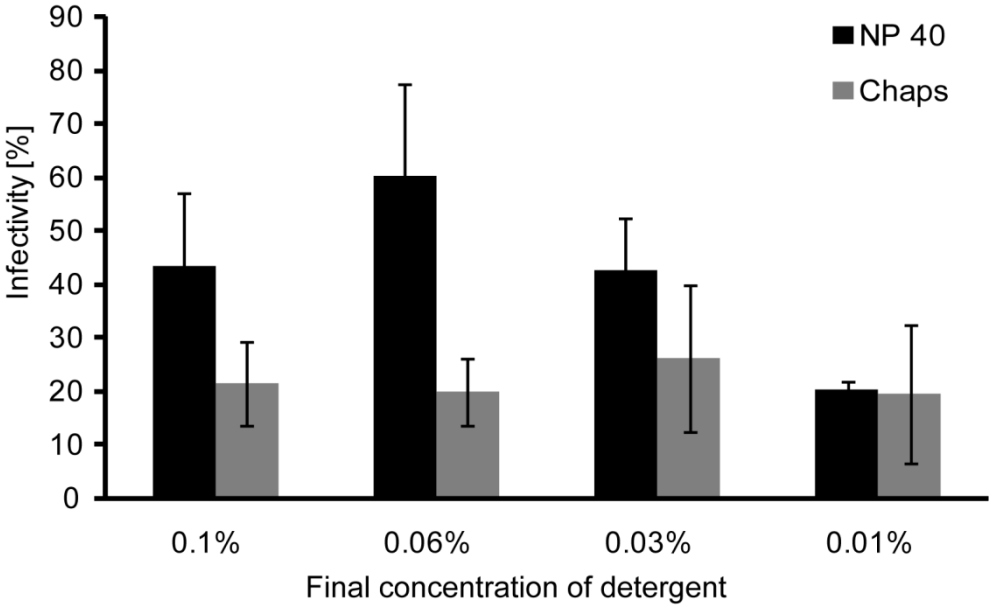


Figure 9: Optimizing detergent concentration for cell lysis in infectivity plates. HeLa cells were infected with C. trachomatis with MOI 0.5 in 96 well plates. 48 hpi cells were lysed with the indicated final concentrations of either Nonidet P40 (black bars) or Chaps (grey bars) detergents and the lysates were used to infect freshly seeded cells. 24 h later cells were fixed and stained with antibody specific to C. trachomatis MOMP and Hoechst 33342. The results represent means ± SD from three independent experiments.

3.1.5 Finding activating and inhibitory controls

Appropriate controls are essential in the design of large scale experiments. Activating and inhibitory controls should be selected to develop a screen assay, as controls give important information on the reproducibility, robustness and ease of the assay.

To find appropriate activating and inhibitory controls we tested siRNA's targeting 10 different genes (Figure 10 a and b) for some of which we had previously observed an influence on Chlamydia infectivity. Also for some of the genes more than one siRNA was tested, which are indicated by number followed by gene name (Figure 10 **Error! Reference source not found.**a and b).

1500 HeLa cells/well were plated with 100 µl of growth medium (GM) in a 96 well plate 24 h before were transfected with the indicated siRNA's (Figure 10 a and b). 3 days post transfection cells were infected with MOI 0.5 of C. trachomatis. The first plate was fixed 24 hpi to analyze the primary infection. The other plate was lysed 48 h after infection and dilutions were used to infect non-transfected HeLa cells to determine the infectivity. After

fixation, cells were stained with Hoechst dye for counting cell numbers and with an antibody directed against the MOMP protein of C. trachomatis. Shown are the inclusion/nuclei of primary infection (Figure 10 a) and infectivity (Figure 10 b). As neutral control transfection, a siRNA for F-Luciferase was used as it does not target any gene in the genome.

Knock down of Arf1, p115 and giantin led to increase in infectivity, small GTPase Arf1 KD resulted in the similar phenotype for all three siRNA sequences tested with least standard deviation (SD). Knock down of MAP1LC3A, MAP1LC3B, MAP1LC3A+B, CD71, Abi1 and Abi2 led to decrease in infectivity, KD of MAP1LC3A+B together resulted in almost complete decrease with least SD.

As shown in Figure 10 c and d, Arf1 knockdown leads to bigger inclusions as well as to a higher number of progeny. In contrast to this, MAP1LC3A/B knockdown results in smaller inclusions and almost no progeny. Automated image analysis of the primary infection, revealed that the inclusion size is increased by 81% upon Arf1 knockdown whereas it decreased after loss of MAP1LC3A/B by 41%. Furthermore, in re-infected plates quantification of inclusions per cell shows a 245% increase with siArf1 and an almost complete loss of re-infection after MAP1LC3A/B knockdown (Figure 10 c and d). These two controls not only show the functionality of our assay but also were used as activating and inhibitory controls throughout the siRNA screen.

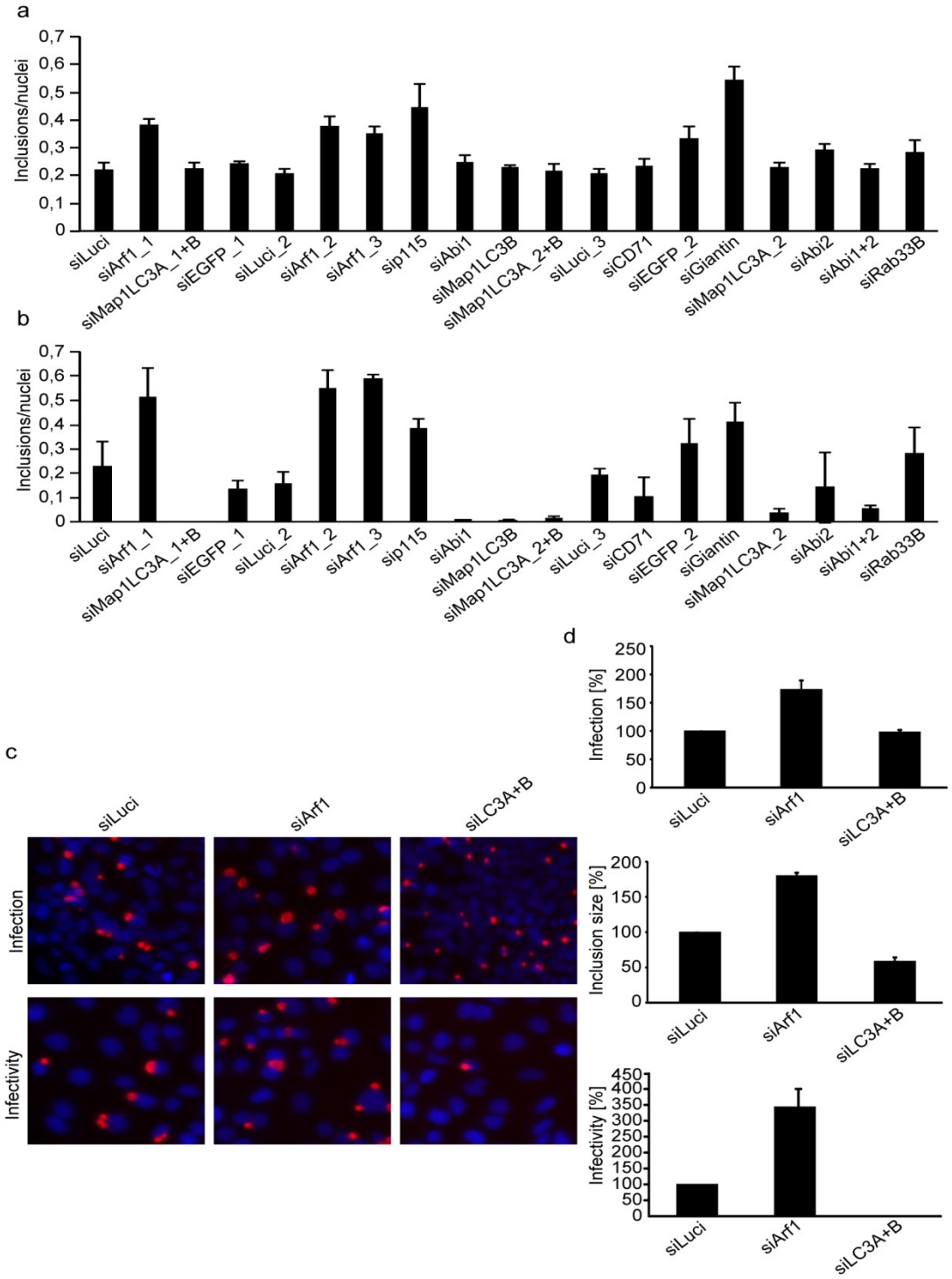


Figure 10: Selection of controls for the assay. HeLa cells were transfected with the siRNAs targeting the indicated genes. For some genes more than one siRNA sequence was tested which are indicated with a number following the siRNA name. Three days post transfection cells were infected with C. trachomatis with MOI 0.5. Shown are the inclusions/nuclei of (a) primary infection and the (b) infective progeny for each siRNA tested. (c) siRNA targeting Luciferase (siLuci), Arf1 (siArf1), and Map1LC3 a +b (siLC3) were established as neutral, activating and inhibitory controls. Shown are representative images. (d) The calculated percentage of infection, inclusion size and infectivity for siArf1 and siLC3 normalized to siLuci. Shown is the mean ± SD of 3 independent experiments.

3.1.6 Optimizing Chlamydia multiplicity of infection

Consequently the optimal multiplicity of infection (MOI) to distinguish changes both in primary infection and the infectivity phenotypes resulting from the KD of target genes was determined. HeLa cells seeded at a density of 1500 cells/well one day before were transfected with the control siRNAs identified in 3.1.5. 3 days post transfection cells were infected with C. trachomatis with MOI 0.25, 0.5, 0.75 or 1. The first plate was fixed 24 hpi to analyze the primary infection. The other plate was lysed 48 h after infection and dilutions were used to infect non-transfected HeLa cells to determine the infectivity. After fixation, cells were stained with Hoechst dye for counting cell numbers and with an antibody directed against the MOMP protein of C. trachomatis.

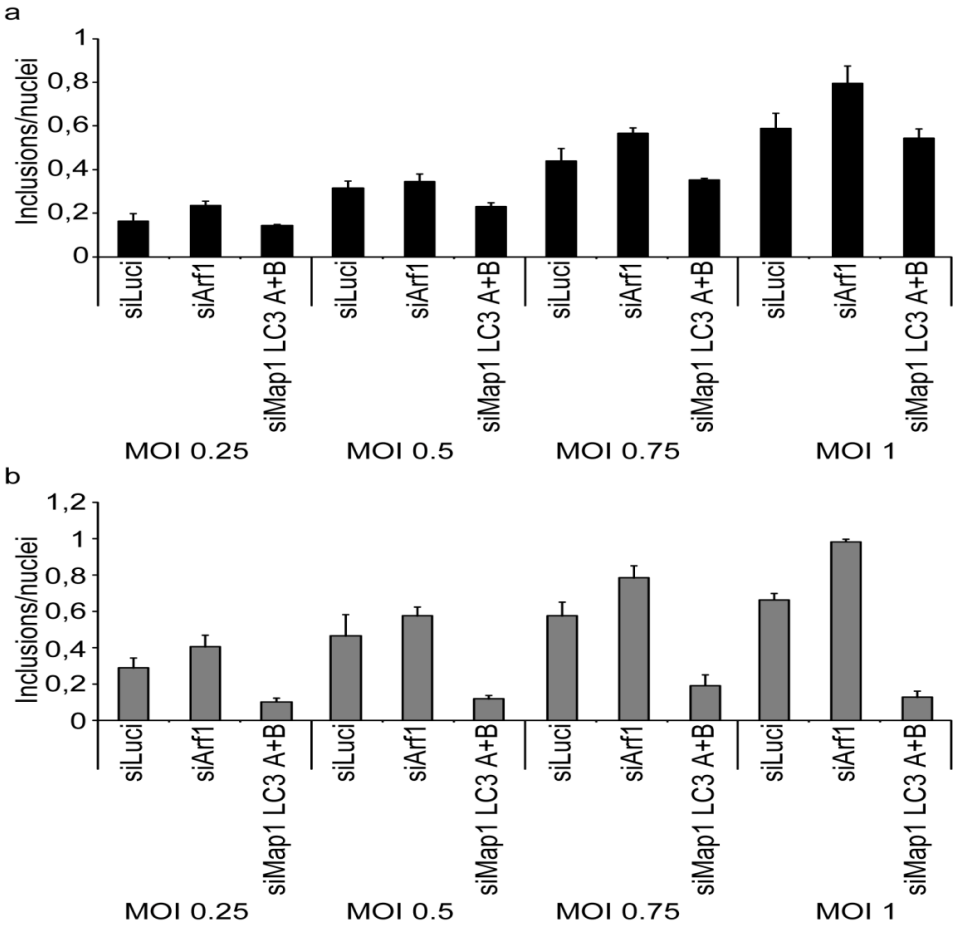


Figure 11: Optimizing MOI of C. trachomatis for infectivity assay in 96 well plates. HeLa cells were transfected with the indicated siRNA and three days post transfection cells were infected with C. trachomatis with the indicated MOI. The primary infection as well as infectivity was measured. Shown are the Inclusions/nuclei for the primary infection (a) and the infectivity (b) for each MOI tested. The results represent means ± SD from three independent experiments.

Nuclei and Chlamydia inclusions were acquired by automated microscopy and images were analyzed using the Scan^R software. MOI of 0.5 showed primary infection rate between 25 to 50% (Figure 11 **Error! Reference source not found.**a) and infectivity between 25 to 75% (Figure 11 **Error! Reference source not found.**b), with the reinfection rate of 50% for neutral control providing enough space to be able to distinguish increase and decrease of infectivity induced by KD of specific genes. The other MOI tested i.e. 0.25, 075 and 1 were either too low or too high. Consequently we decided to use MOI of 0.5 for the screen.

3.1.7 Titration of cell number

The starting cell numbers seeded for the assay would be equal irrespective of the siRNA to be transfected. However, siRNA transfection can induce cytotoxicity or cell proliferative effects to different extent depending on the targeted gene resulting in differences in the cell numbers between samples. Therefore it was essential to determine the range of cell numbers with in which the assay would work normally without having an influence on the infectivity outcome. For this purpose HeLa cells were seeded at different densities starting from 1000 to 64000 cells/well in 100 µl of growth medium in 96 well plates in duplicates 1 day before infection. Cells were infected with same amount of C. trachomatis. The first plate was fixed 24 hpi to analyze the primary infection. The other plate was lysed 48 hpi and dilutions were used to infect non-transfected HeLa cells to determine the infectivity, which was also fixed 24 h post reinfection. After fixation, cells were stained with Hoechst dye for counting cell numbers and with an antibody directed against the MOMP protein of C. trachomatis. Nuclei and Chlamydia inclusions were acquired by automated microscopy and images were analyzed using the Scan^R software. Nuclei number and inclusion/nuclei of primary infection (Figure 12 a and c) as well as nuclei number and inclusion/nuclei of infective progeny (Figure 12 b and d) were determined. The results of the inclusion/nuclei of primary infection as well as infectivity show that the assay works well within the tested range of cell numbers without having a profound influence on the infective progeny (Figure 12 d). However, very low as well as very high cell numbers can have an influence on the infectivity outcomes as the inclusion development can be different, with inclusion being bigger with low cell density and smaller with higher cell densities. Hence, we decided that in

the screen if the total cell numbers counted for each sample from 4 microscopic fields were less than 500 the result for that gene should not be considered for further evaluation.

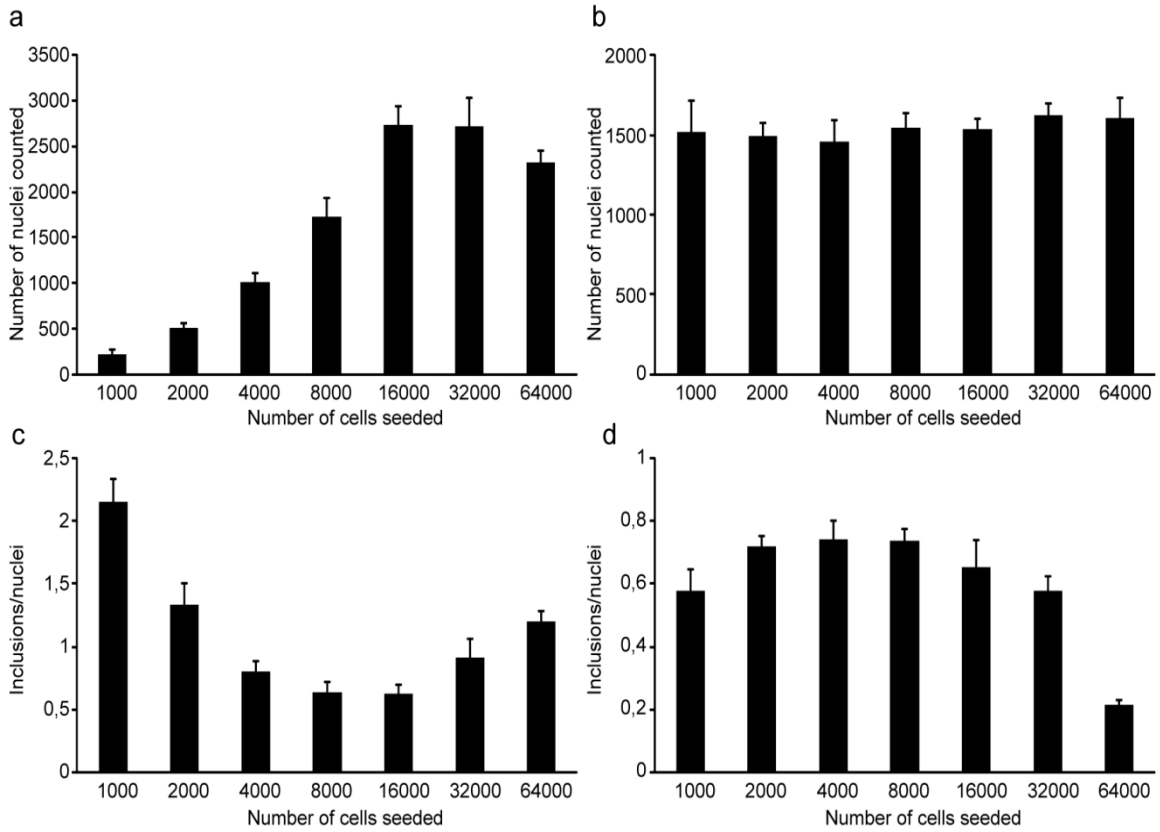


Figure 12: Titration of cell number. To determine the range of cell numbers within which the screen can be performed and to establish the right cell numbers for screen, HeLa cells were seeded in 96 well plates at different cell numbers and infected with same amount of C. trachomatis. The primary infection as well as infectivity was measured. Shown are the nuclei counts (a) and the inclusion/nuclei (c) of primary infection and nuclei counts (b) and inclusion/nuclei (d) of the infectivity plates respectively. The results represent means ± SD from three independent experiments.

3.1.8 Automation of the assay

Since the assay was eventually intended for high throughput screening, it was necessary to minimize the number of steps which require manual intervention. So the next step in the establishment of the assay was to automate the liquid handling steps for the process of transfection and infectivity. Initial cell seeding and the primary infection step of the assay were performed manually. For this purpose, a pipeting robot (BioRobot® 8000 system, Qiagen) was used.

The process of transfection involves several steps, such as removal of medium from the cells, preparation of siRNA and HiperFect complexes in 96-well plates and pipetting the complexes

into the appropriate wells. The process of infectivity involves addition of Nonidet P40 to the cells, mixing of the detergent and the medium as well as shaking the plate to ensure effective cell lysis, performing serial dilution of the lysate and finally pipetting the 1:100 diluted lysate into appropriate wells of the target plate after medium aspiration.

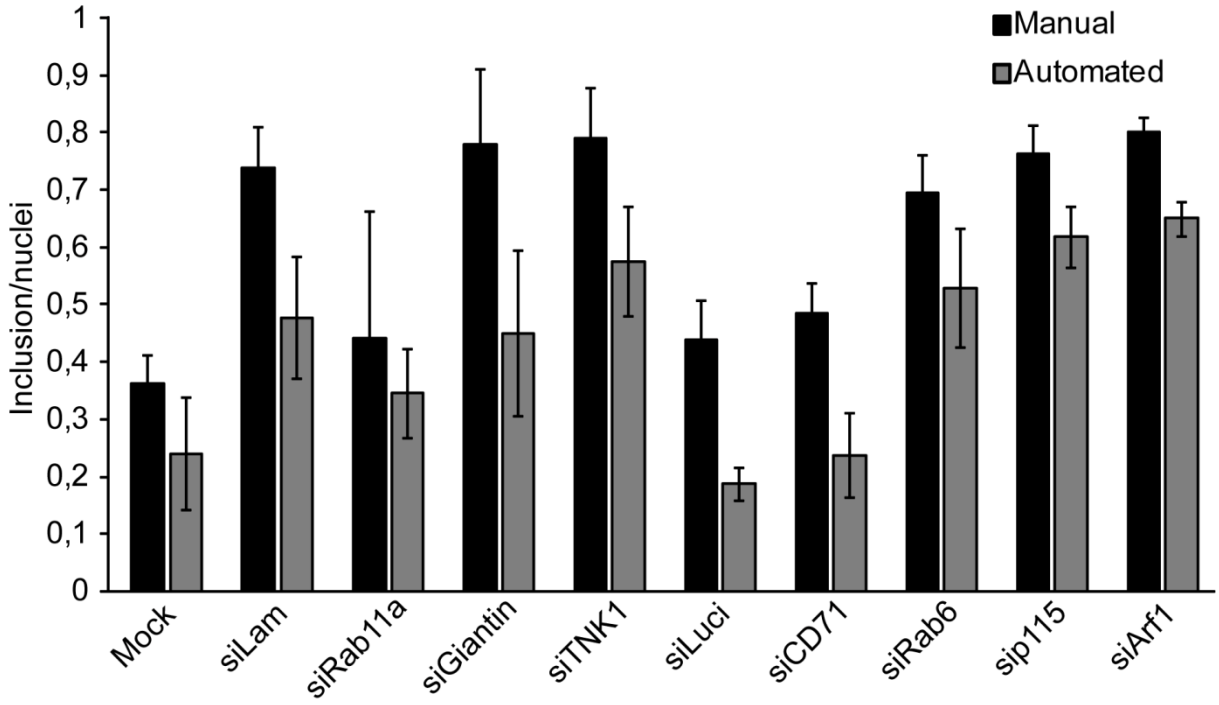


Figure 13: Automation of the assay. HeLa cells were either transfected manually or using BioRobot® 8000 system (Qiagen) with the siRNA targeting the indicated genes. Three days post transfection cells were infected with C. trachomatis with MOI 0.5. Following this cell lysis and reinfection was also either performed manually or using BioRobot® 8000 system (Qiagen). Shown are the inclusion/nuclei of the infectivity plates from manual (black bars) or automated (gray bars) protocols. The results represent means ± SD from three independent experiments.

Because the assay was initially established manually, automation was optimized for each step separately, hence it was necessary to compare between the manual and automated assay before commencement of the screen.

For this HeLa cells were transfected with siRNAs indicated in Figure 13 either manually or using the BioRobot® 8000 system (Qiagen). 3 days post transfection both were manually infected with C. trachomatis using automated multichannel pipette followed by infectivity performed 48 hpi again either manually or using the BioRobot® 8000 system (Qiagen).

Although the observed inclusion/nuclei of the automated protocol (Figure 13 gray bars) were less than that of the manual protocol (Figure 13 black bars) for all the tested samples, the results displayed the same tendency in both. These results led us to conclude that the

automation of the assay to be successful and robotic protocols can thus be used for screening.

3.2 The siRNA screen

I screened three siRNA libraries: A kinase library targeting 646 kinases and kinase binding proteins, an apoptosis library directed against 418 apoptosis related genes and a custom library targeting 461 genes with a broad range of cellular functions. The kinase and the apoptosis library contained two siRNAs per gene which have been pooled into one well. For the custom library, if available, the two siRNAs also have been pooled into one well. During assay development, we observed that cells in the outer wells of a 96 well plate tended to accumulate at the outer edge of the plate resulting in unequal cell density compared to inner wells. Since infection efficiency might depend on the cell density, we omitted these wells from the screen. Thus, the screening plates contained siRNAs against 48 library genes and a quadruplicate of the three types of controls: siLuc (neutral), siMAPLC3A/B (inhibitory), and siArf1 (activating) (Figure 14**Error! Reference source not found.**). Each of the library plates was screened at least three times. Only plates where the controls showed increased re-infection rates upon Arf1 knockdown and a stark decrease after MAP1LC3A and MAP1LC3B knockdown were chosen for further analysis.

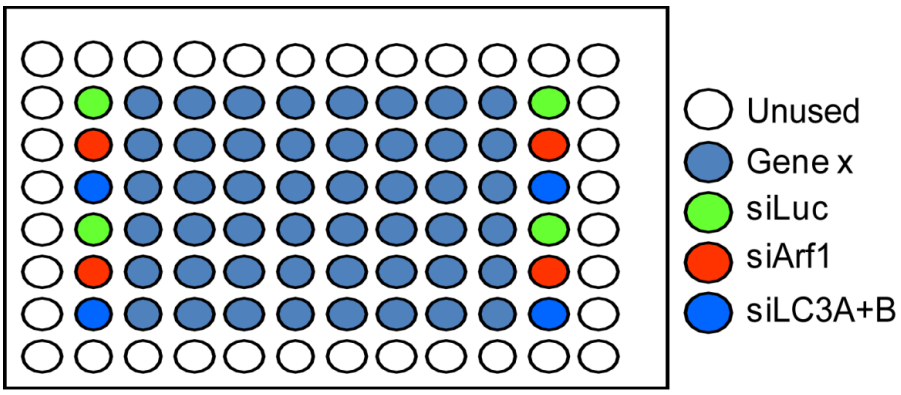


Figure 14: Overview of the plate layout and distribution of siRNA controls and targets. Cells were seeded in the outer wells, but were left unused to avoid edge effects.

3.2.1 Quality control of the primary screening data

As a first step in the data analysis quality control of the data was performed. For this a plate to plate correlation coefficient matrix was generated for each parameter of the assay. Figure 15 **Error! Reference source not found.**shows the correlation matrix of the infectivity readout from apoptosis library as an example of the analysis. The matrix was generated by plotting the correlation coefficients of all plate pairs. As can be seen from the plot only the repeats plates containing same set of siRNA were highly correlated, whereas plates containing different siRNA sets are much less correlated (Figure 15**Error! Reference source not found.**). Through this analysis we could ensure that the data generated to be specific due to the gene perturbations introduced by siRNA. The correlation shown among only the repeats also indicates reproducibility of the assay irrespective of when the experiment was performed. For further analysis, only 3 repeat plates with highest correlation were chosen.

3.2.2 Normalization of the primary screening data using POC and B-Score

To rule out the concerns related to positional effects of wells within plates and to minimize false-positive and false-negative rates, two distinct statistical normalization methods were performed with the obtained infection and infectivity data. They are percent of control analysis (POC) (done in collaboration with Dr. Johannes Schuchhardt, Microdiscovery) and B-Score analysis (Brideau, et al., 2003) (done in collaboration with Dr. Klaus-Peter Pleissner).

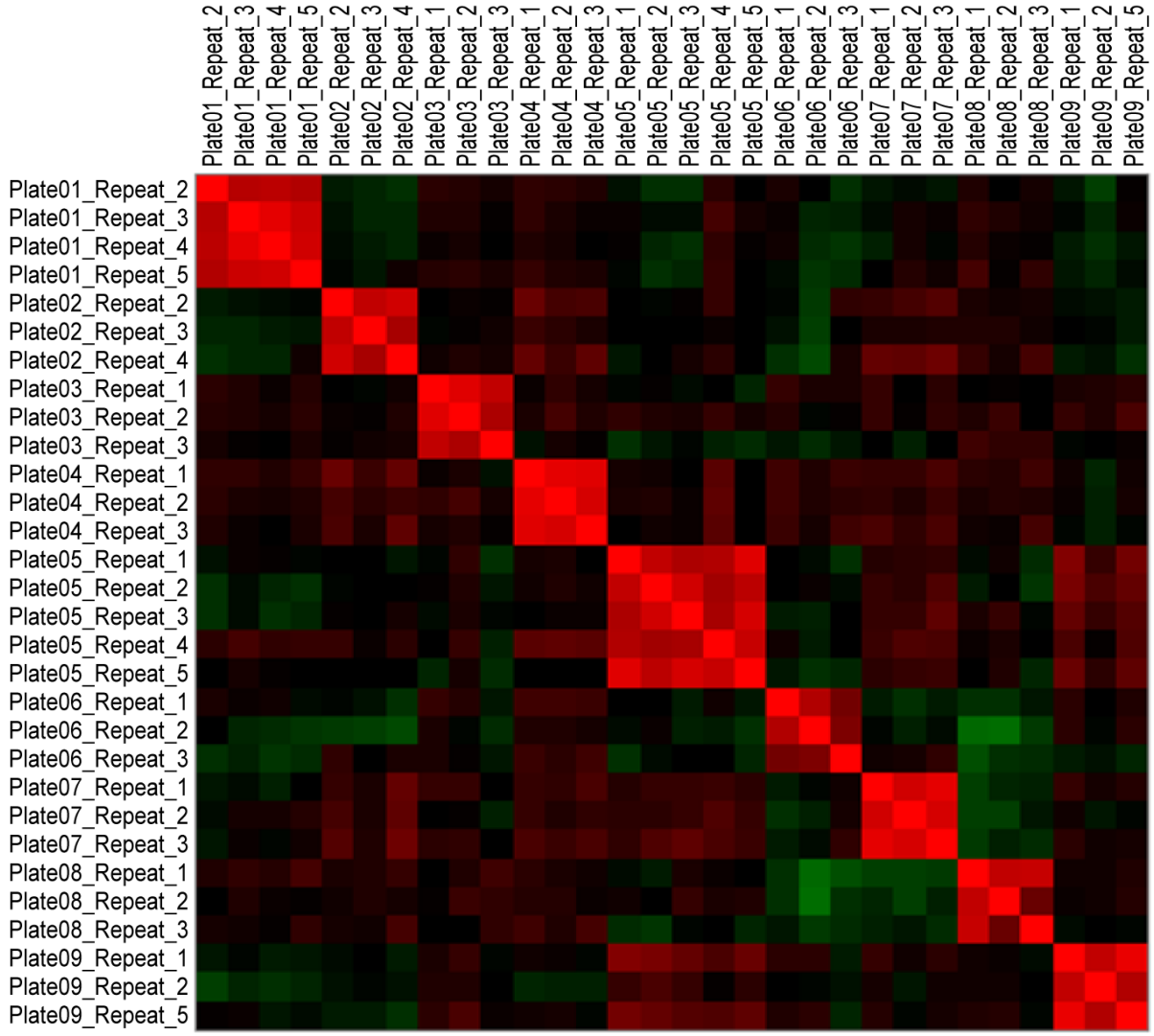


Figure 15: Correlation plot for plate wise quality control. The correlation coefficient matrix was calculated plate wise, based on all controls and samples. Red colors indicate correlation, green color anticorrelation between plates. Plate repeats containing the same set of siRNA showed good correlation values, whereas for plates containing different siRNA sets no correlation was observed. The infectivity correlation matrix for one sub-library is displayed as an example for the whole screen.

Percent of control' is one processing method that attempts to correct for plate to plate variability by normalizing compound measurements relative to controls. In this method, the raw measurements for each compound is divided by the average of within the plate controls (Malo, et al., 2006). Therefore, in the percent of control (POC) analysis method, the data selected from the correlation analysis was subjected to two step normalization. For example, the normalization steps for the inclusion counts of one library are shown in Figure 16. In the first step of the normalization, data from individual plates were normalized to mean of the Luciferase control (Figure 16 a) using the following formula

POC= Xi/C

Where, Xi is the raw measurement on the ith compound and C is the mean of the measurements of the control. In the second step, the normalized values were scaled and centered for the repeat groups based on the median of all normalized values of the plate (Figure 16 b).

The B score is a robust analysis method, which uses an index of dispersion that is more resistant to the presence of outliers and more robust to differences in the measurement error distributions of the compounds (Malo, et al., 2006) in the following way.

First, the residual (rijp) of the measurement for row i and column j on the pth plate is obtained by fitting a two-way median polish as defined below

$$r_{ijp} = y_{ijp} - \hat{y}_{ijp} = y_{ijp} - (\mu + \check{R}_{ip} + \hat{C}_{jp})$$

The residual is defined as the difference between the observed result (y_{ijp}) and the fitted value ($\hat{y}_{ijp}$, defined as the estimated average of the plate (μ_p) + estimated systematic measurement offset for row i on plate p ($\check{R}_{ip}$) + estimated systematic measurement column offset for column j on plate p ($\hat{C}_{jp}$)). For each plate p, the adjusted median absolute deviation (MADp) is obtained from the r_{ijp}'s (MADp). The B score is then calculated as follows:

$$\text{Bscore} = r_{ijp} / \text{MADp}$$

A two-way median polish is first computed to account for row and column effects of the plate. The resulting residuals within each plate are then divided by their median absolute deviation to standardize for plate-to-plate variability. The B score has three advantages: it is nonparametric (that is, makes minimal distributional assumptions), it minimizes measurement bias due to positional effects and is resistant to statistical outliers. The resulting data from both the analysis methods was used for further analysis and hit classification.

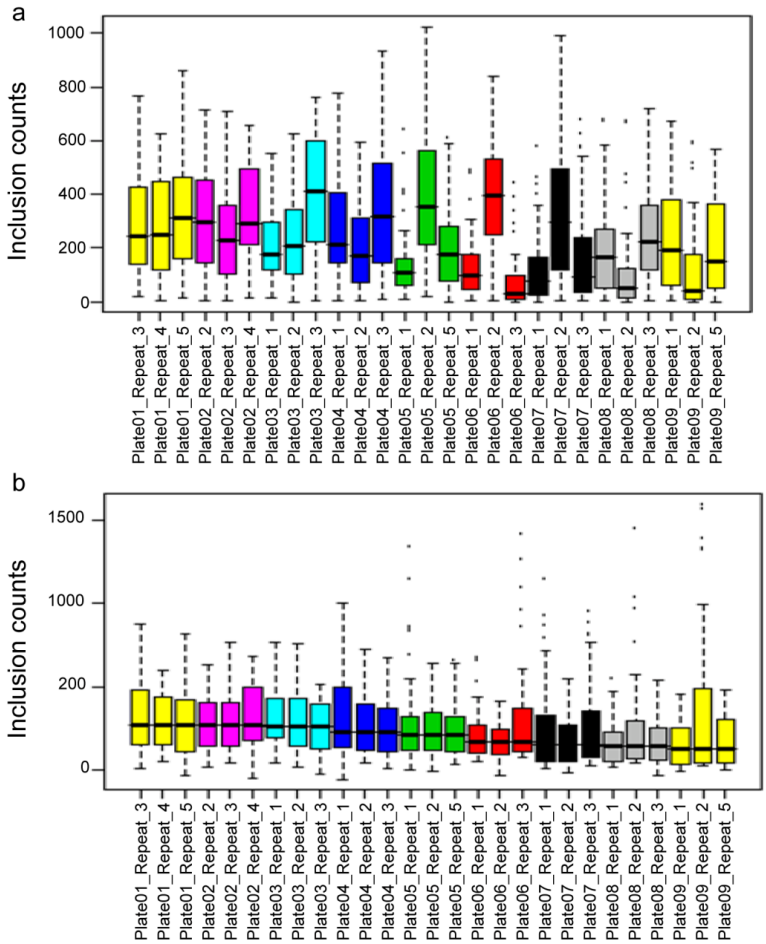


Figure 16: POC two step normalization. Shown are the two normalization steps used in the percent of control (POC) analysis of the primary screen. (a) Plates were normalized using the mean of the Luciferase controls on each plate and (b) the normalized values were scaled and centered for the plate repeats containing the same set of siRNAs based on the median of all normalized values of the plate. Plate repeats are depicted in the same color. Plates from one sub-library are displayed as an example for the whole screen.

3.2.3 Statistical analysis and hit classification of the primary screen

Two different statistical methods were used for the analysis of the primary screen data with two different normalization methods described in 3.2.2. One of the methods was the cellHTS software package implemented in Bioconductor/R developed specifically to analyze cell-based high-throughput RNAi screening data. Data was normalized using the B-Score normalization available within the package. As a next step in the analysis, hits were scored by transforming the normalized measurements into z-scores for their statistical significance. This step calculates a single number, the Z score, for each sample. Hits were then classified by defining thresholds of the z-score for both up and down regulating phenotypes, which were 3 and -1 respectively. A total of 204 targets were identified as primary hits with this method (Figure 17 B-score).

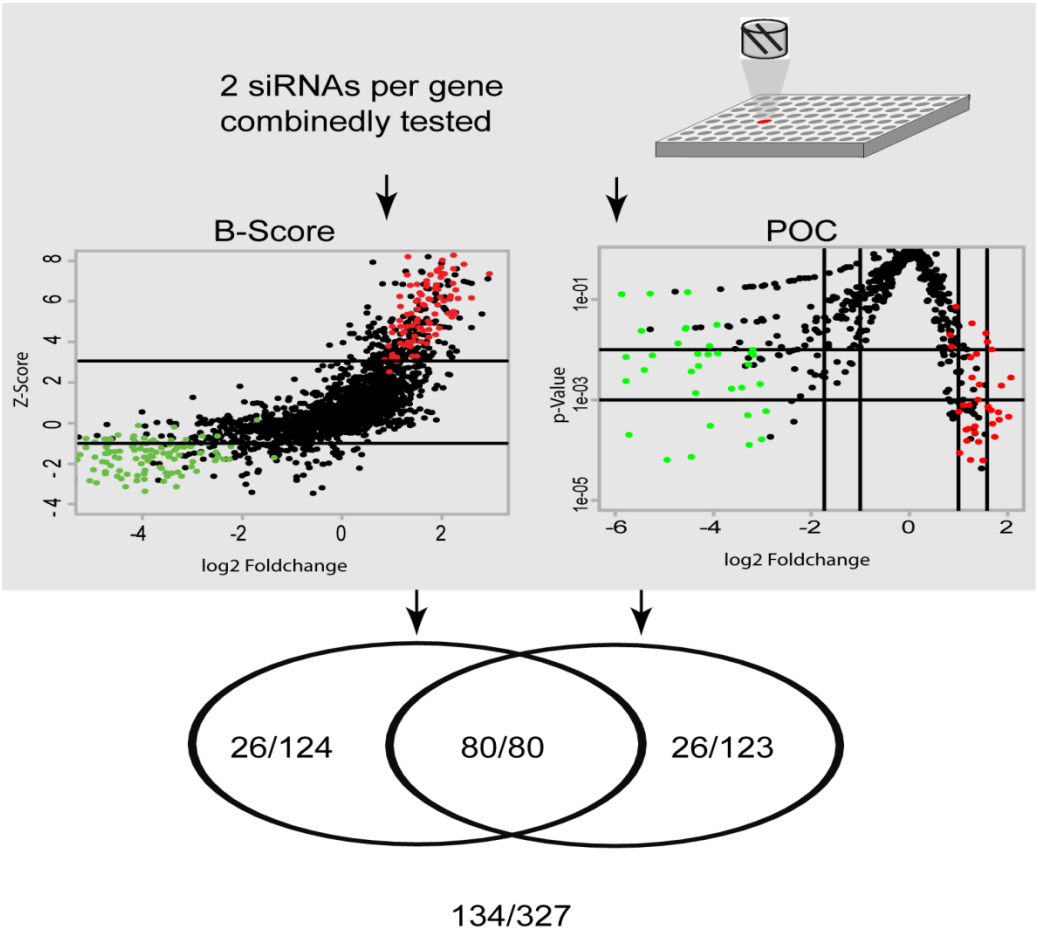


Figure 17: Statistical analysis for the primary screen. Infectivity data of cells transfected with a pool of two siRNAs targeting one specific gene were analyzed in parallel using two statistical normalization methods; B-Score and percent of control (POC). Negative controls are marked in green, positive controls in red and samples in black. Lines indicate the defined thresholds used for primary hit definition. All 80 overlapping primary hits from both statistical analysis methods and 26 non-overlapping primary hits exclusively from each B-Score and POC methods were chosen for hit validation

The second statistical method was custom designed in which percent of control normalization was used. To measure the statistical significance of the data p-values were calculated for each of the sample. Also the log2 ratios of infectivity were calculated. Volcano plot was generated by plotting the log2 ratios and calculated p-values on X and Y axis respectively. Hits were then classified by defining p-value and fold change thresholds. A total of 203 targets were identified as primary hits with this method (Figure 17 POC). Comparison of the hits scored from each of the methods revealed 80 targets to be overlapping in both methods. For hit validation all 80 overlapping primary hits from both statistical analysis methods and 26 non-overlapping primary hits exclusively from each B-Score and POC methods were chosen (Figure 17).

3.2.4 Correlation analysis of the primary screening data

Next I analyzed the effect of primary infection on the cell numbers. The counted nuclei of the transfected but uninfected (TO) plate were plotted against the counted nuclei of the primary infection (TI) plate for all samples The analysis showed a high correlation between the two sample groups, implying that the infection with Chlamydia does not have profound effect on the cell numbers (Figure 18 **Error! Reference source not found.**a).

Next we analyzed, whether the primary screen hits are biased by changes in cell number due to the knockdown of the target genes. The counted nuclei number of the TO plate and the log2 fold change of infectivity were plotted against each other. Further the primary hits were marked in another color to observe distribution of the hits in relation to the variation in the cell number caused by knockdown of the target genes (Figure 18 b). Both the up and down regulating primary hits distributed throughout the plot without any specific correlation to the cell numbers. This clearly shows that changes of the cell number caused by siRNA transfections has no influence on the infectivity outcome and the primary hits identified are a result of specific target gene knockdown.

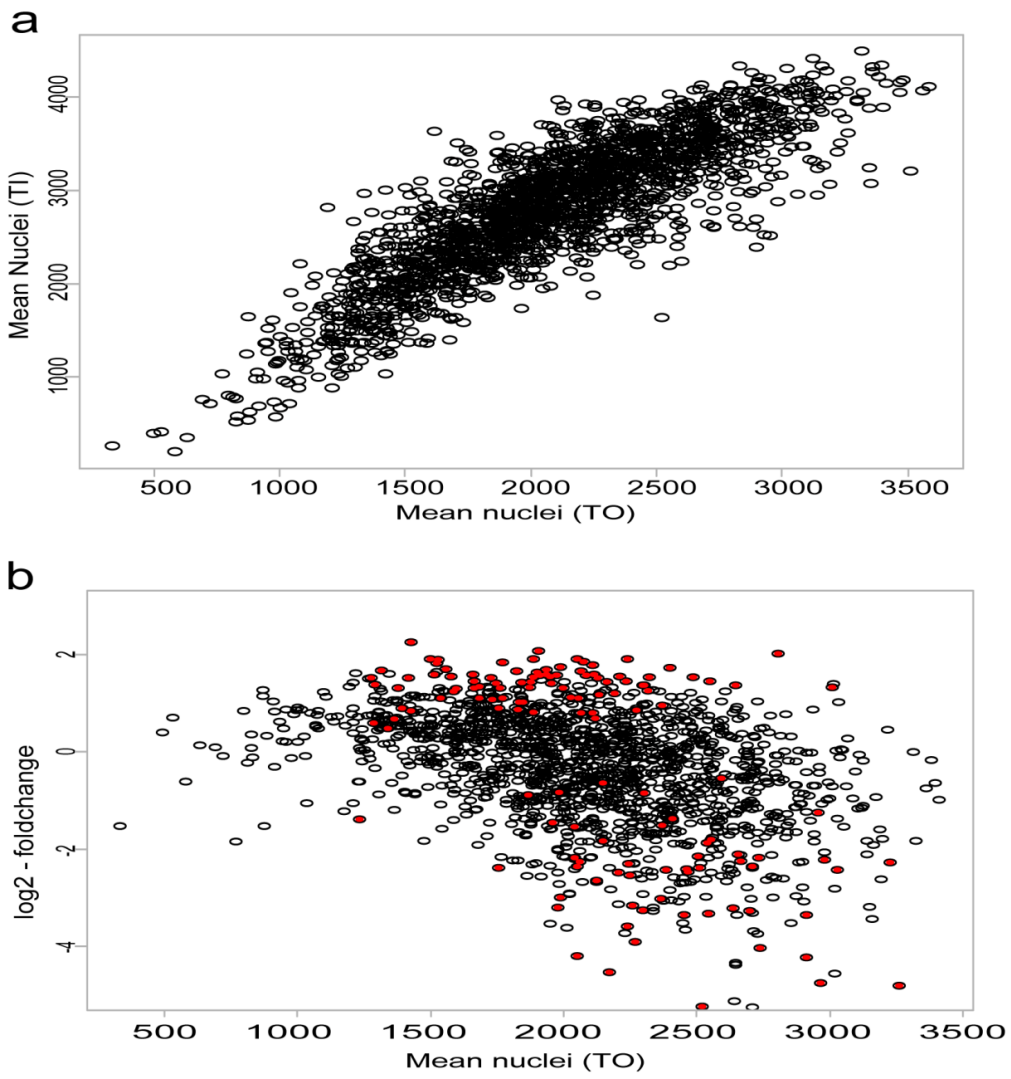
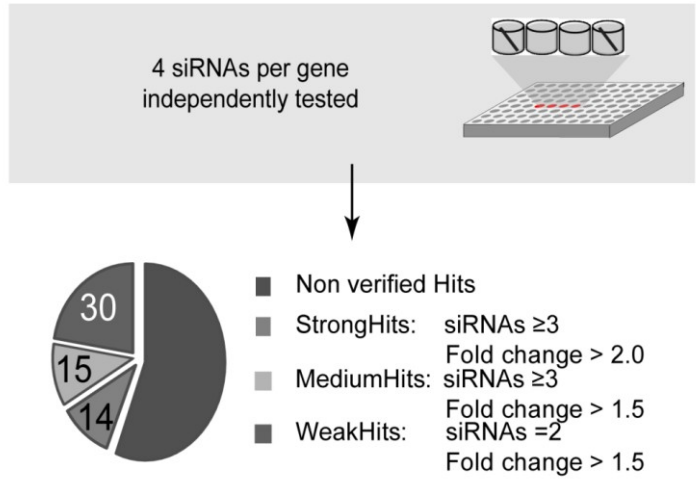


Figure 18: Correlation analysis. Cell distribution and distribution of hits (a) Shown is the correlation plot of the raw values of the nuclei counts for the transfected plates without infection (TO) on X axis versus the nucleus counts of the transfected and infected plates (TI) on the Y axis. (b) Correlation plot of the nuclei counts for the transfected plates without infection (TO) on X axis versus the log2-fold change of the infectivity measurements. The primary hits are highlighted in Red.

3.2.5 Statistical analysis and hit classification from the hit validation

The data from the hit validation was analysed with the POC method described in 3.2.2 and the final hits defined in to three categories in the following way: Strong hits: 2 fold change in the observed phenotype in the same direction as observed in the primary screen with at least 3 of the 4 siRNAs tested. Medium hits: 1.5 fold change in the observed phenotype in the same direction as observed in the primary screen with at least 3 of the 4 siRNAs tested. Weak hits: 1.5 fold change in the observed phenotype in the same direction as observed in the primary screen with at least 2 of the 4 siRNAs tested (Figure 19 **Error! Reference source not found.**a).

a



b

	B-Score	OL	POC
Strong	3	6	5
Medium	1	11	3
Weak	5	21	4

Figure 19: Statistical analysis for the hit validation data and comparison of analysis methods. (a) Hit validation was performed for 134 primary hits using 4 independent siRNAs targeting one specific gene. Data was analyzed using POC normalization in a similar way as the primary screen. Final hits were clustered into strong, medium and weak hits according to the biological effect and the number of positive siRNAs. (b) Number of final hits scored from each analysis methods used in the primary screen, as well as the overlapping genes is shown.

Among the final hits 9 were the number of validated strong, medium and weak hits obtained with the B-score method accounting to 35% validation rate, 12 were the number of validated strong, medium and weak hits obtained with the POC method accounting to 46% validation rate and 38 were the number of strong, medium and weak hits obtained overlapping hits of the primary screen accounting to 48% validation rate (Figure 19 b). From this it is clear that the POC method for has slightly higher rate of validation rate compared to B-score method. A list of the all the validated hits is shown in the Table 1

Note: The hits list table also depicts from which analysis method the target was chosen from in the primary screen in the last column, C=Overlaps, P=POC method and B=B-Score method.

Table 1: Final Hit List

Hit Category	Gene ID	Name	Phenotype	Hit fromAnalysis method

Strong	5728	PTEN	Down	C
	1315	COPB1	Down	B
	1028	CDKN1C	Down	P
	2261	FGFR3	Down	C
	1432	MAPK14	Up	B
	56616	DIABLO	Up	C
	2987	GUK1	Up	C
	2869	GRK5	Up	C
	5894	RAF1	Up	P
	23043	TNIK	Up	P
	5747	PTK2	Up	C
	3845	KRAS	Up	P
	8887	TAX1BP1	Up	B
	387	RHOA	Up	C
Medium	4293	MAP3K9	Down	P
	5608	MAP2K6	Down	C
	9516	LITAF	Down	B
	65266	WNK4	Up	C
	701	BUB1B	Up	C
	1607	DGKB	Up	C
	26574	AATF	Up	C
	6300	MAPK12	Up	C
	9020	MAP3K14	Up	C
	51347	TAOK3	Up	P
	23591	APR	Up	C
	9891	NUAK1	Up	C
	5245	PHB	Up	C
	7525	YES1	Up	C
	5570	PKIB	Up	P
Weak	330	BIRC3	Down	C
	378	ARF4	Down	P

	1152	CKB	Up	P
	1196	CLK2	Down	B
	1198	CLK3	Down	B
	1716	DGUOK	Up	C
	2932	GSK3B	Down	C
	3678	ITGA5	Up	C
	4055	LTBR	Up	C
	4843	NOS2A	Up	C
	5255	PHKA1	Up	C
	5742	PTGS1	Down	C
	5930	RBBP6	Up	C
	7083	TK1	Up	C
	7422	VEGFA	Down	P
	8396	PIP5K2B	Up	C
	8761	PABPC4	Down	C
	8772	FADD	Down	C
	8784	TNFRSF18	Up	B
	8833	GMPS	Down	C
	9371	KIF3B	Up	C
	10293	TRAIP	Up	C
	10645	CAMKK2	Down	B
	22900	CARD8	Down	B
	65975	STK33	Up	C
	79109	MAPKAP1	Up	C
	79646	PANK3	Up	C
	80271	ITPKC	Down	C
	83549	UCK1	Up	C
	118788	PIK3AP1	Up	P

3.2.6 Ingenuity pathway analysis of the hits

Further, the validated hits were subjected to a pathway analysis to identify signaling pathways which are potentially influencing Ctr infection and infectivity. For this purpose, I used Ingenuity Pathway Analysis software (IPA), a web based interface, which builds interactions based on literature evidences (done in collaboration with Dr. André P. Mäurer). The strong, medium and weak validated hits were independently subjected to a functional analysis using IPA. This program grouped the validated hits according to proposed molecular and cellular functions. Functional groups significant for the Fisher`s exact p-value in all three hit categories, were identified to be involved in cell death, cell morphology, cell cycle, cell signaling, cell to cell signaling, cellular assembly, small molecule biochemistry, cellular movement, post translational modification and amino acid metabolism. All other possible functions that did not meet the significance criteria were not considered for further analysis.

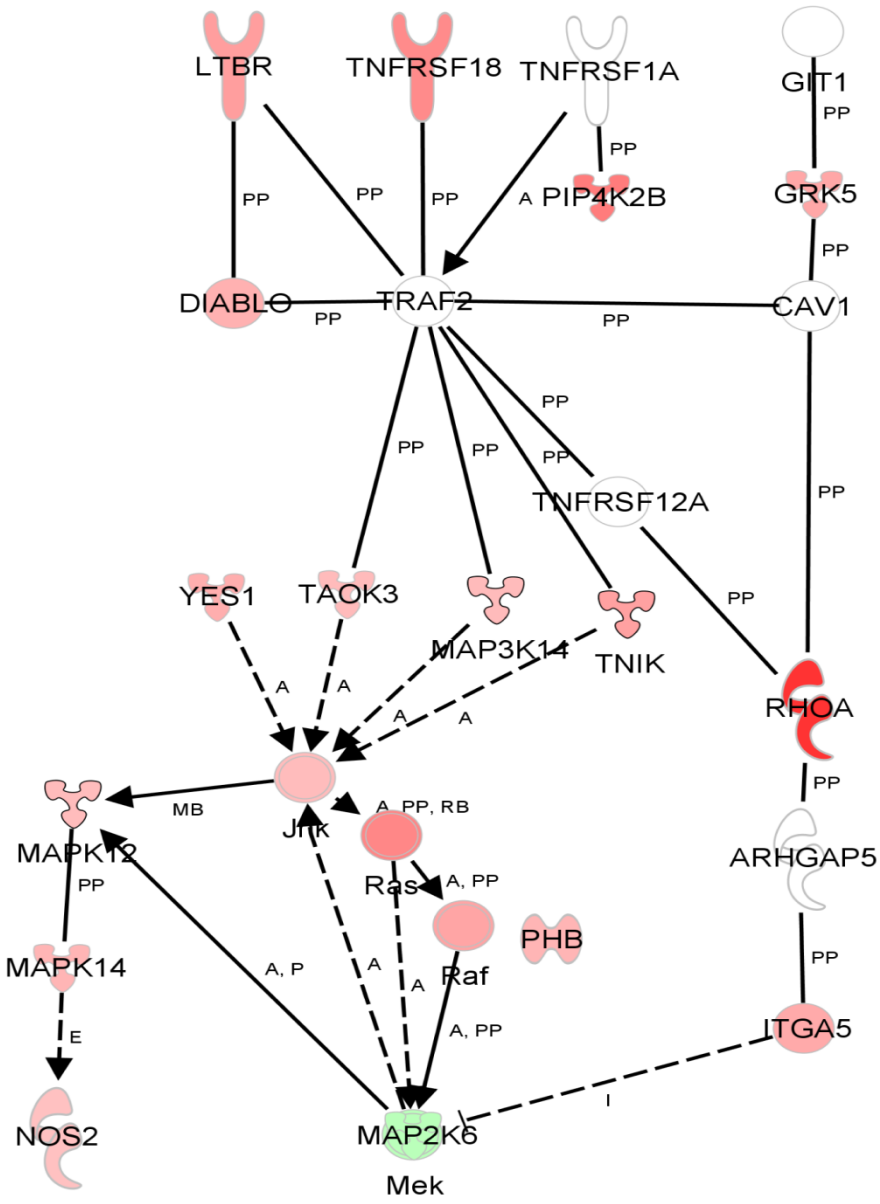


Figure 20: Pathway analysis. The validated hits were analyzed using Ingenuity Pathway Analysis (IPA) software. These hits were revealed to be involved in several molecular functions. From the identified functions, hits were subjected to network analysis. Shown is the simplified network based on the interactions predicted by IPA. Hits are designated in colors based on infectivity phenotypes: green for down regulation and red for up regulation. Other proteins (non-hits) were added to complement the networks which are displayed in white. The connections between the proteins are depicted with letters: A= Activation, PP=protein protein interaction, E= Expression, RB= Phosphorylation, MB=Member, I=inhibition. The solid lines show direct and the dashed lines show indirect interactions.

Further, the hits from these groups were used to develop network using IPA, based on protein-protein interaction, activation, inhibition and transcription. Several possible signaling pathways were identified from this analysis. Interestingly, Ras and Raf-1 were found to have a central role in the downstream signaling in all the identified networks. Further, the complex networks were curated to simplify into one single network for better clarity (Figure

20). Specifically, Ras and Raf-1 depletion was found to increase Ctr infectivity in the screen. This was seemingly in contradiction to previous evidences (Su, et al., 2004). Therefore, it is interesting to elucidate the role of Ras and Raf-1 in Chlamydia infections.

3.3 Functional characterization

3.3.1 Time course analysis of the Chlamydia infected cells

A previous publication has described the activation of the Ras-Raf-Mek-Erk pathway after Ctr infection leading to the activation of cPLA2 by phosphorylation (Su, et al., 2004). This study primarily employed chemical inhibitor to inhibit Mek and Raf1 and showed that Mek inhibition leads to decrease Ctr infectivity as well as of cPLA2 phosphorylation. However, our screening results strongly suggested that Ras and Raf knockdown led to increased Ctr infectivity. Therefore we decided to compare the screening results and literature by blocking the Ras-Raf-Mek-Erk pathway with chemical inhibitors as well as siRNA.

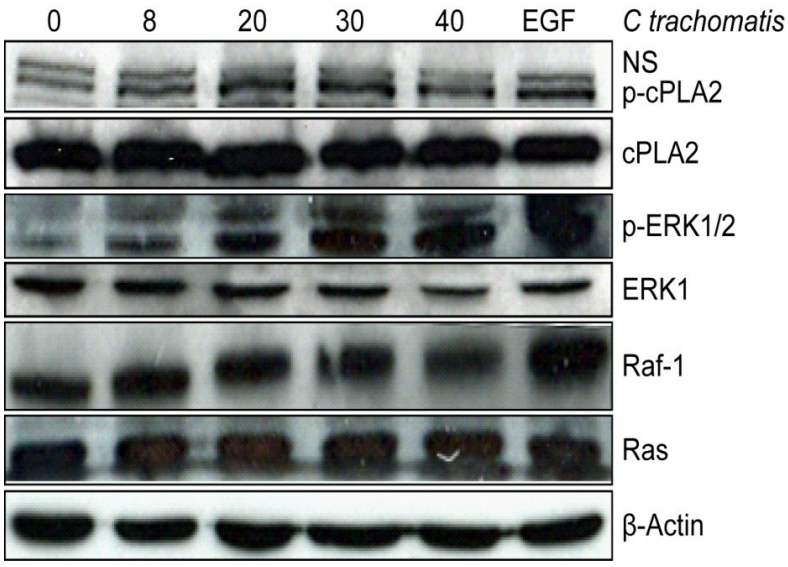


Figure 21: Time course analysis of Ctr infected HeLa cells. HeLa cells infected with MOI 3 of Ctr, were harvested at the indicated time points after infection and subjected to immunoblot analysis for Ras, Raf, Erk, cPLA2, phospho-ERK (pERK1/2) and phospho-cPLA2 (p-cPLA2). β-actin was used as loading control. A representative blot of three independent experiments is shown

To determine the optimal time point of infection to study the regulation of the Ras/Raf/Mek/Erk pathway and cPLA2 activation upon Chlamydia infection, a time course analysis was performed.

HeLa cells seeded in 6 well plate one day before infection were infected with MOI 3 of C. trachomatis. Samples were harvested by adding SDS sample buffer directly to the wells after 0, 8, 20, 30, 40 hpi. As a positive control for the activation of Erk pathway cells were treated with 100 ng/ml of EGF for 5 minutes before harvesting the cells similar to infected cells. Samples were then boiled for 5 minutes before subjecting to western blot analysis of Ras, Raf1, Erk, p-Erk, cPLA2 and p-cPLA2 using specific antibodies. As expected, HeLa cells show strong phosphorylation of Erk1/2 and cPLA2 starting from 8h until 44h post infection. However the levels of Ras, Raf1, Erk and cPLA2 remained unchanged throughout the infection time course (Figure 21**Error! Reference source not found.**). As the previous studies employed the 30h post infection time point and also because strong up-regulation of the p-Erk1/2 and p-cPLA2 was observed in our study at the same time point, we decided to use this time point of infection for further evaluation.

3.3.2 Mek Inhibitor U0126 leads to decrease in Erk and cPLA2 phosphorylation and Chlamydia infectivity

To study the effect of Mek inhibition upon treatment with the chemical inhibitor U0126, HeLa cells seeded in a 6 well plate one day before infection and were infected with MOI 3 of C. trachomatis. 2 hpi medium was replaced with fresh IM containing either 10 or 100µM U0126 inhibitor or left without treatment and the inhibitor was maintained throughout the infection duration. 30 hpi samples were harvested and subjected to western blot analysis of Ras, Raf1, Erk, p-Erk, cPLA2 and p-cPLA2. Duplicates of the samples treated identically were used for determination of infectivity as described in 5.2.5.2.

HeLa cells showed strong phosphorylation of Erk1/2 and cPLA2 at 30h post Ctr infection, whereas infection in the presence of the MEK inhibitor U0126 led to reduced phosphorylation of Erk as well as of cPLA2 (Figure 22 a). Consistently, treatment with U0126 also led to decreased infectivity compared to mock treated and infected cells (Figure 22 **Error! Reference source not found.**b). This is very much in agreement with the earlier publication (Su, et al., 2004), implying that Mek inhibition using U0126 leads to decrease in the Erk phosphorylation and subsequent cPLA2 activation.

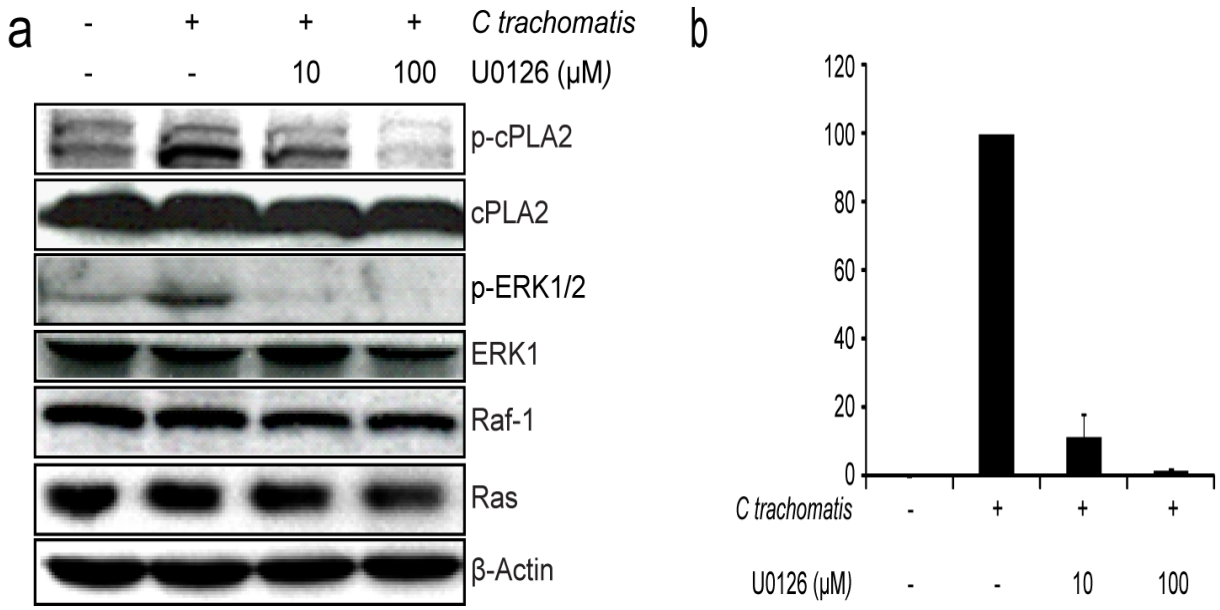


Figure 22: Inhibition of Mek by Chemical inhibitor U0126 leads to decrease in Erk and cPLA2 phosphorylation and Chlamydia infectivity. (a) Non infected and Ctr infected HeLa cells with and without UO126 inhibitor treatment, were harvested 30h pi and subjected to immunoblot analysis for Ras, Raf, Erk, cPLA2, phospho-ERK (pERK1/2) and phospho-cPLA2 (p-cPLA2). -actin was used as loading control. A representative blot of three independent experiments is shown. (b) Non infected and Ctr infected HeLa cells with and without the inhibitor UO126 were lysed 48h post infection and used to reinfect freshly seeded cells. These cells were fixed 24h post reinfection and the infectivity quantified using automated microscopy. Shown are the ±SD of three independent experiments normalized to mock treated infected cells.

3.3.3 Knockdown of Mek1/2 by siRNA also leads to decrease in Erk1/2 phosphorylation

Next I analyzed whether the knockdown of Mek1/2 also has same effect on Erk activation as observed with the chemical inhibitor U0126. HeLa cells seeded in 12 well plate one day before were transfected either with siRNA targeting Mek 1 and Mek2 together (siMek1/2) or siRNA targeting Luciferase (siLuci) was used a transfection control. One day later the cells were detached and reseeded into 6 well plates and incubated further 48 hours. Three days post transfection the cells were infected with MOI3 of C. trachomatis. Samples were harvested 30 hpi subjected to western blot analysis of, p-Erk, and Mek.

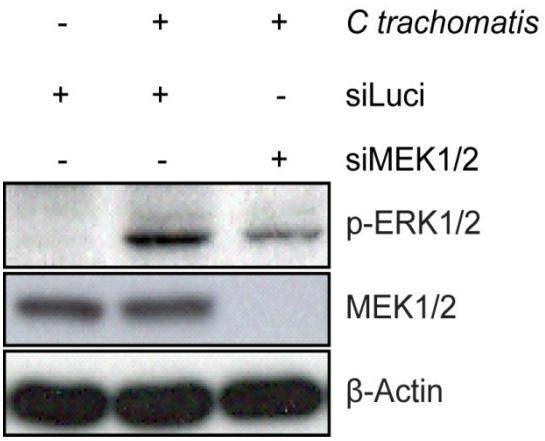


Figure 23: Knockdown of Mek1/2 leads to decrease in Erk phosphorylation. Non infected and Ctr infected. HeLa cells with siRNA targeting Luciferase (siLuci) and MEK1/2 (siMEK1/2) were harvested 30 hpi and subjected to Western blot analysis for MEK1/2 and pERK. β-actin was used as loading control. A representative blot of three independent experiments is shown.

These results show an increase of Erk phosphorylation after Ctr infection which decreased almost to the basal level in Mek1/2 KD cells (Figure 23). In the primary screen we also observed a clear decrease in the infectivity after Mek knockdown (data not shown). This suggests that the KD of Mek using siRNA has the same effect as the chemical inhibitor U0126 in downstream activation of Erk, implying the requirement of Mek for activation of Erk and its downstream effectors in Chlamydia infection

3.3.4 Activation of Erk after Chlamydia infection does not depend on Ras and Raf1, and knockdown of Ras and Raf1 leads to increased infectivity

Knockdown of Ras or Raf1 consistently led to increase in Chlamydia infectivity in the screen and were subsequently identified as strong hits from the hit validation. However, it was published that the inactivation of Raf1 by chemical inhibitor GW5074 reduces the activation of Erk and cPLA2 (Su, et al., 2004). To clarify the discrepancies from the published data and our results, we decided to further examine the involvement of Ras and Raf1 by siRNA based gene knockdown.

After knockdown of Ras and Raf1 (described in 5.2.9), subsequently HeLa cells were infected with MOI 3 of C. trachomatis (described in 5.2.4). Samples were harvested 30 hpi and were subjected to western blot analysis of, Ras, Raf1, Erk, p-Erk, cPLA2 and p-cPLA2 (described in 5.2.13)

In contrast to the observation of Mek1/2 inhibition by chemical inhibitor and siRNA, Ras or Raf1 knockdown did not decrease the Erk and cPLA2 phosphorylation (Figure 24 **Error! Reference source not found.**a). This clearly suggests that Ras and Raf1 are not involved in activation of Erk in Chlamydia infection. It was also observed that knockdown of Ras or Raf1 led to increase in the Chlamydia infectivity compared to Luciferase transfected samples (Figure 24 b) contrary to that observed with Mek inhibition implying that knockdown of either Ras or Raf1 offers growth advantage to Chlamydia within the host cells.

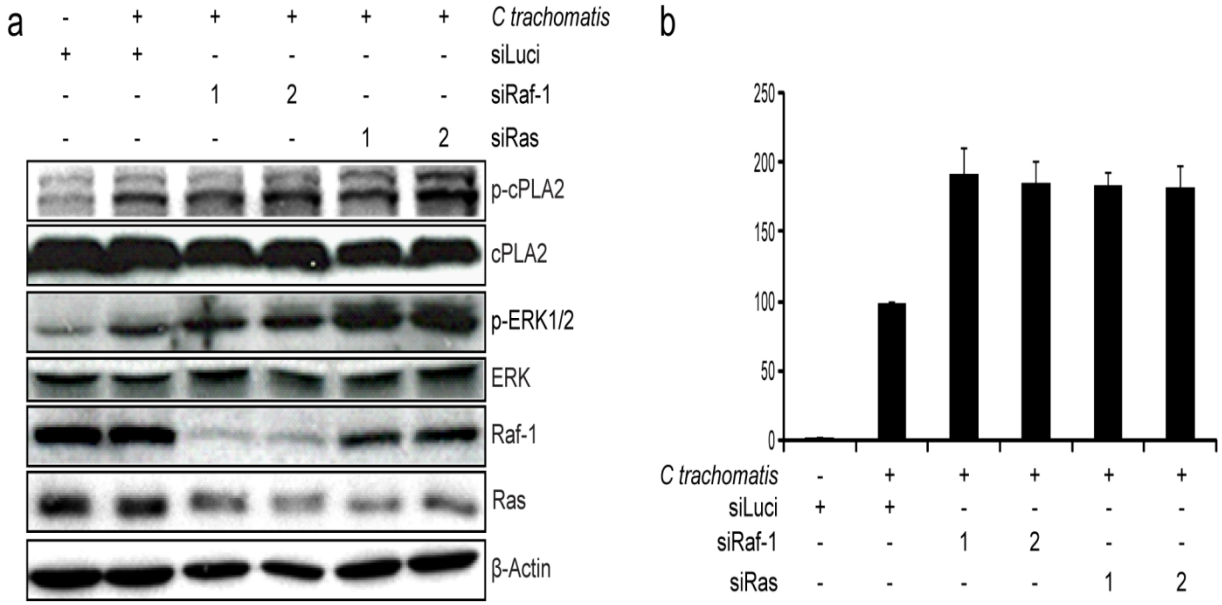


Figure 24: Effects of Ras and Raf1 knockdown on ERK and cPLA2 phosphorylation and Chlamydia infectivity. (a) Non infected and Ctr infected HeLa cells with siRNA targeting Luciferase, Ras and Raf1 were harvested 30h pi and subjected to Western blot analysis for Ras, Raf1, Erk, cPLA2, p-ERK1/2 and p-cPLA2. For Ras and Raf1 knockdown two independent siRNAs were used indicated as 1 and 2. β-actin was used as loading control. A representative blot of three independent experiments is shown. (b) Non infected and Ctr infected HeLa cells with and without siRNA targeting Luciferase, Ras and Raf1 were lysed 48h post infection and used to reinfect freshly seeded cell. For Ras and Raf1 kockdown two independent siRNAs were used indicated as 1 and 2. These cells were fixed 24h post reinfection and the infectivity quantified using automated microscopy. Shown are the ±SD of three independent experiments normalized to infected and siLuci transfected cells.

3.3.5 Raf1 is inactivated by Ser259 phosphorylation after Chlamydia infection in an AKT1/2 dependent manner

My results thus far show that Erk phosphorylation is Raf1 independent after C. trachomatis infection. Phosphorylation of Ser259 has been reported to inactivate Raf1 (Rommel, et al., 1996; Zimmermann and Moelling, 1999), therefore I investigated if Raf1 phosphorylation is influenced in Chlamydia infection.

HeLa cells were transfected with siRNA targeting Luciferase (siLuci) and Akt 1 and 2 together (siAkt 1+2), 3 days post transfection cells were infected with MOI 3 of C. trachomatis. Samples were harvested 30 hpi and were subjected to western blot analysis of Akt, phospho-Erk and phospho-Raf1 (Ser259) (described in 5.2.13). Chlamydia infected cells showed an increased phosphorylation of Raf1 at Ser259, confirming the hypothesis. However, knockdown of Akt led to decreased Raf1 phosphorylation to the basal uninfected levels, implying that Akt1/2 is required for phosphorylating Raf1 at Ser259 (Figure 25). On the other hand, increased phosphorylation of Erk upon Chlamydia infection was not affected by knockdown of Akt (Figure 25). Taken together this data strongly suggests that Chlamydia can inactivate Raf1 by Akt dependent phosphorylation at Ser259.

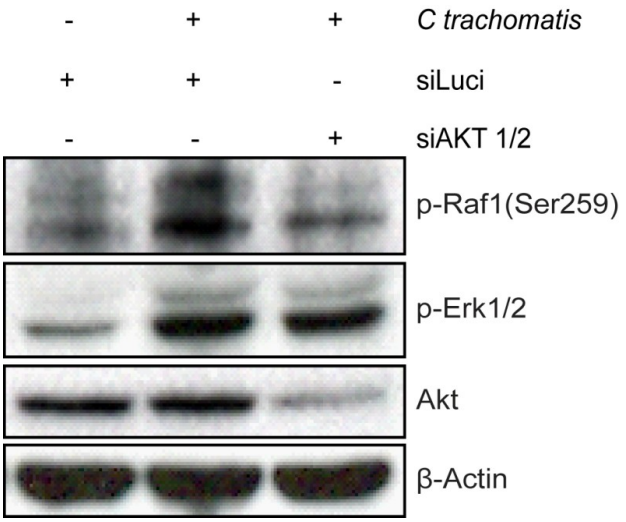


Figure 25: Inactivation of Raf1 at Serine 259 after Ctr infection depends on AKT. Non infected and Ctr infected HeLa cells with siRNA targeting Luciferase (siLuci) and Akt (siAkt1+2) were harvested 30 hpi and subjected to Western blot analysis for Akt, phospho-Erk and phospho-Raf1 (Ser259). □-actin was used as loading control. A representative blot of three independent experiments is shown.

3.3.6 Raf1 and phospho Raf1 are redistributed to the Chlamydia inclusion and co-localize with 14-3-3β

Upon Akt dependent Ser259 phosphorylation, Raf1 is able to bind to 14-3-3β (Muslin, et al., 1996; Zimmermann and Moelling, 1999). In addition during infection 14-3-3β has been shown to be recruited to the Chlamydia inclusion (Scidmore and Hackstadt, 2001). Therefore I investigated how the cellular localization of Raf1 is affected by Chlamydia infection.

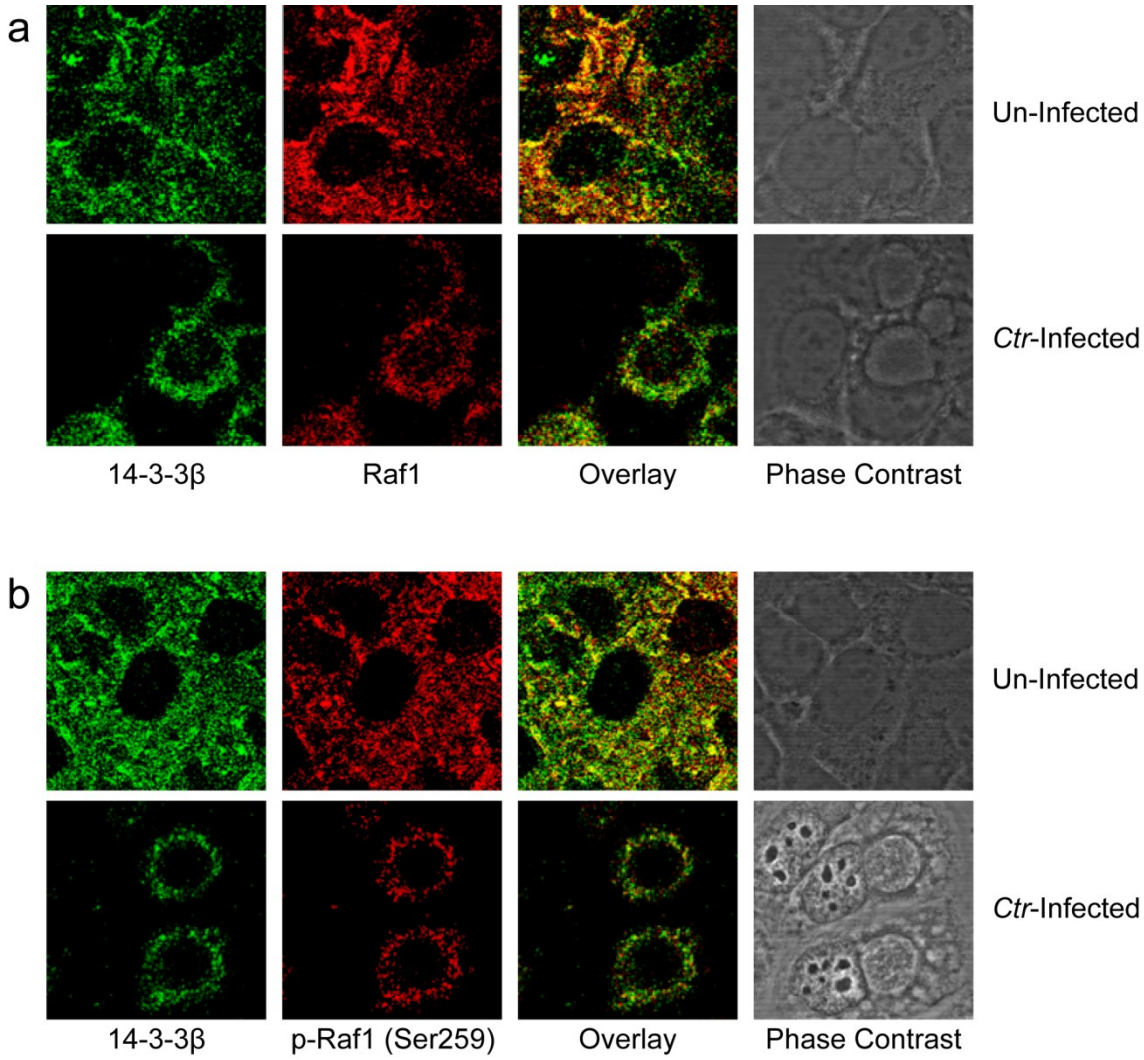


Figure 26: Raf1 and phospho Raf1 are redistributed to the Chlamydia inclusion. Non infected and Ctr infected HeLa cells fixed 30h pi, stained for 14-3-3 beta and either (a) Raf1 or (b) phospho-Raf1. Images were acquired with a confocal microscope. Overlay and phase contrast images are shown to demonstrate the co-localization and Chlamydia inclusion respectively. Representative images of three independent experiments are shown.

To determine the cellular localization of Raf1 and p-Raf1 (Ser259) in infected cells confocal microscopy was used. HeLa cells infected with an MOI 2 of C. trachomatis were fixed 30 hpi. The samples were then stained with antibodies targeting Raf1and 14-3-3β or p-Raf1 (Ser259) and 14-3-3β.

In uninfected cells both Raf1 and p-Raf1 (Ser259) were observed dispersed throughout the cytoplasm with no specific pattern of accumulation. The same pattern of distribution was also observed for 14-3-3β (Figure 26 a and b top panel). However, in Chlamydia infected cells Raf1 and p-Raf1 (Ser259) were observed in close proximity to the chlamydial inclusion (Figure 26 a and b bottom panel). The pattern of distribution of Raf1 and p-Raf1 (Ser259) was found to be similar to that of 14-3-3β. Furthermore, the overlay of Raf1 and p-Raf1 with 14-3-3β from respective samples demonstrated a high degree of co-localization of both the

proteins at the inclusion membrane. This clearly indicates that upon infection with C. trachomatis the Raf1 is re-localized to the inclusion membrane in a 14-3-3β dependent manner.

3.3.7 Redistribution of Raf1 to the sub cellular membrane fraction after Chlamydia infection depends on Akt and 14-3-3β

To further confirm Raf1 re-localization to the inclusion membrane and the involvement of 14-3-3β and Akt in this process. I performed a sub cellular fractionation experiment of uninfected and Ctr infected cells with knockdown of either Luciferase, 14-3-3β or Akt as described in 5.1.7.1.

In uninfected cells with Raf1 was found being equally distributed between the cytosolic and the membrane fractions, whereas after infection it was predominantly found in the membrane fraction implying that Raf1 is redistributed to Chlamydia membrane fraction (Figure 27 **Error! Reference source not found.**a). Uninfected cells with Akt knockdown showed a similar distribution pattern as cells with Luciferase knockdown for Raf1 (Figure 27 b).

In contrast, after Akt knockdown Raf1 was detected equally in the membrane and cytosolic fraction in Chlamydia infected cells. This clearly suggests that Raf1 redistribution to the membrane fraction is dependent on Akt mediated phosphorylation of Raf1 at Ser259 (Figure 27 b).

In comparison to luciferase knockdown, uninfected cells with 14-3-3β knockdown showed a prevalent distribution of Raf1 in the cytosolic fraction (Figure 27 c). Also in infected cells, 14-3-3β knockdown did not lead to a redistribution of Raf1 to the membrane fraction (Figure 27 c), proving the impact of 14-3-3β on Raf1 redistribution.

Altogether, the data strongly suggests that Raf1 is recruited to the Chlamydia inclusion in an Akt and 14-3-3β dependent manner.

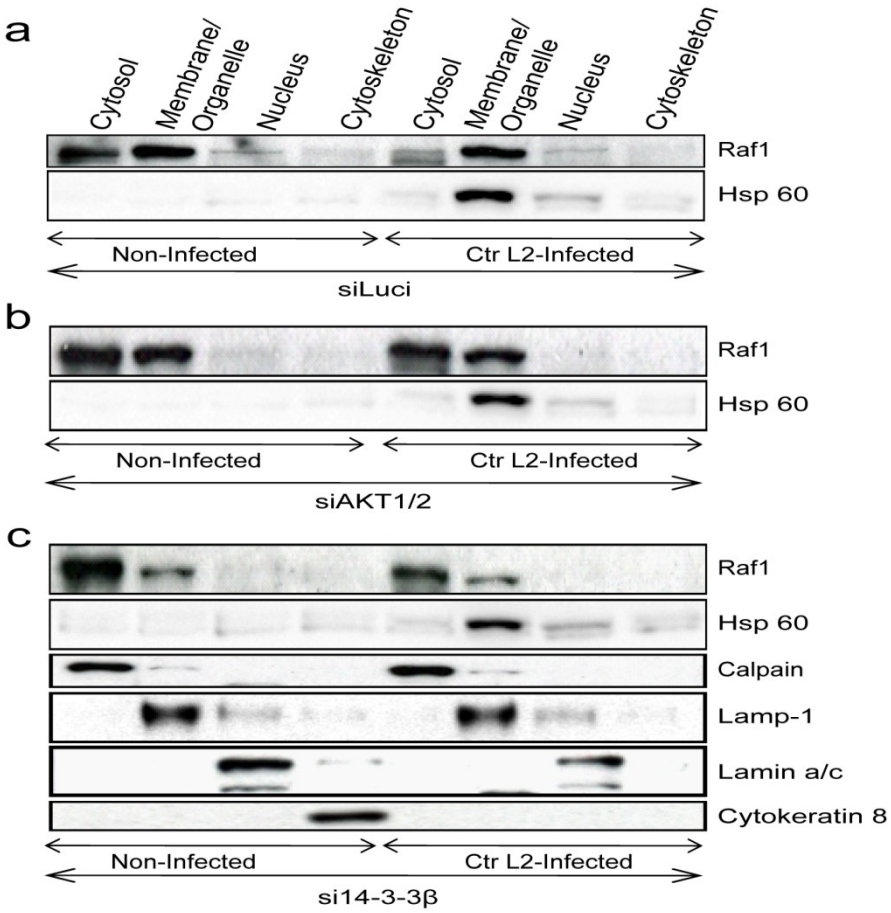


Figure 27: Redistribution of Raf1 to membrane fraction depends on Akt and 14-3-3β. Non infected and Ctr infected HeLa cells treated with siRNA targeting (a) Luciferase (b) Akt1/2 or (c) 14-3-3β were separated into sub cellular fractions using Sub cellular Proteome Extraction Kit (Calbiochem) 30h post infection and subjected to Western blot analysis for Raf1 and chlamydial Hsp60. Calpain, Lamp1, Lamin a/c and Cytokeratin8 were used as markers for cytosolic, membrane/organelle, nuclear and cytoskeletol sub cellular fractionations. The sub cellular fractionation markers were found to be similarly distributed in all the conditions; therefore only from the 14-3-3β samples are shown as an example.

4 Discussion

Pathogenic bacteria have evolved various strategies to create a niche for replication inside the host. For instance, as an obligate intracellular pathogen, Chlamydiae rely on host cells for all aspects of their survival, from the initial attachment with the host cell membranes, to cellular invasion, acquisition of host cell metabolites and intracellular replication (Coombes, et al., 2002). Despite that with the available experimental approaches thus far several molecular mechanisms of the chlamydial pathogenesis have been elucidated, however the overall processes underlying the infection are only partially understood.

The ease and availability of RNAi technique to inactivate gene expression in the eukaryotic cells provides the opportunity to uncover the novel host-pathogen interactions which might otherwise be overlooked.

Therefore my study involves RNAi screening with Chlamydia in human cells, which is the first functional RNAi screen to date with a possibility to identify host cell factors important for Chlamydia throughout its complete developmental cycle including entry in to host cells, survival, replication and re-differentiation in to RBs. Establishment of such a screen has to deal with several parameters ranging from standardizing cell culture seeding procedures to handling of large amounts of highly infectious bacteria for successful establishment of synchronous infection in 96 well formats (Echeverri, et al., 2006). Therefore I have carefully optimized the infection procedure which would allow me to monitor both increase and decrease in primary infection and infective progeny as a consequence of target gene knockdown through siRNA (Figure 8**Error! Reference source not found.**). Cell lysis conditions using detergent concentration, where it efficiently lyses cells releasing all the infectious Chlamydia particles without any influence on the target cells of the infectivity plate (Figure 9), were also optimized. Identification of activating and inhibitory controls enabling me to monitor the functionality of the assay throughout the screen (Figure 10) was crucial for the successful screening. Optimum Chlamydia multiplicity of infection with a range, so that changes in the infection and infectivity resulting from knockdown of target genes could be monitored (Figure 11) was established. I also established optimal cell numbers allowing me to score phenotypes for most of the siRNAs tested. With these conditions, I established a robust and specific assay, described in detail in section 3.1.1. This semi-automated, highly sensitive and robust assay combined with sophisticated statistical algorithms for analysis

enabled me to perform an unbiased screen to identify host cell factors involved in the entry and survival of Chlamydia in host cells.

In the past few years several RNA interference screens have been performed in Caenorhabditis elegans, Drosophila and human cells. These screens have shed light on various cellular processes such as cell viability (Boutros, et al., 2004), cytokinesis (Eggert, et al., 2004), Wnt Signaling (DasGupta, et al., 2005), JAK/STAT Signaling (Baeg, et al., 2005), and mechanisms of host–pathogen interaction, including Listeria and Mycobacterium pathogenesis (Agaisse, et al., 2005; Cheng, et al., 2005; Philips, et al., 2005), Candida albicans phagocytosis (Stroschein-Stevenson, et al., 2006), and L. pneumophila exploitation of the early secretory pathway (Dorer, et al., 2006) as well as to identify host proteins that are required for viral entry and replication (Brass, et al., 2008; Supekova, et al., 2008). Specifically there have been two studies focusing on Chlamydia using RNAi screens in Drosophila SL2 cells. One of the screens identified the Abl kinase and PDGFR Signaling to be involved in C. trachomatis entry of host cells (Elwell, et al., 2008) and the other revealing the involvement of the Tom complex in Chlamydia infections (Derre, et al., 2007). Although these studies have uncovered new Chlamydia host cell interactions, they are limited in their ability to monitor the processes involved throughout the developmental cycle of Chlamydia infection in the host cells. Though there are some experimental evidence concerning use of Drosophila SL2 cells as a model for Chlamydia infections, however it does not completely mimic the human host conditions as Chlamydia cannot complete their developmental cycle in these cells (Elwell and Engel, 2005). In contrast to the Chlamydia screens in Drosophila SL2 cells an RNAi screen in HeLa cells, in which Chlamydia can undergo a complete life cycle provides much deeper insights into the interplay between the host and pathogen.

When it was initially discovered, RNA interference was considered as a revolutionary tool, both because of its ease and of its specificity. But relatively shortly after the application of RNAi human cells, a concern was raised about off-target effects. Therefore, in order to ensure the phenotypes observed in the primary screen, 132 hits were validated with additional siRNAs. Similar phenotypes obtained with several siRNAs targeting the same genes therefore more likely to rely on the loss of target gene function than on off-target effects.

It was suggested that only genes that show a perfect complementation to a siRNA would be targeted, and mismatches of just one nucleotide would be sufficient to disrupt this effect

(Elbashir, et al., 2001). However, more recently, several reports suggested that this rule is not always applying and that expression of several unintended genes is also affected by the use of a given siRNA (Birmingham, et al., 2006; Jackson, et al., 2003; Lin, et al., 2005; Ma, et al., 2006). One possibility could be that siRNA molecules can be processed in the cells like the endogenous miRNAs. The human genome, as well as the genomes of other organisms encodes genes generating RNA stem loop structures (Bartel, 2004; Kim and Nam, 2006). These molecules are then successively processed by Drosha and Dicer, two RNaseIII-like enzymes generating an imperfect siRNA-like duplex (Lee, et al., 2003). Conversely to siRNAs that induce cleavage of their mRNA targets (Elbashir, et al., 2001; Hammond, et al., 2000), miRNAs are mainly responsible for translational repression (Bartel, 2004; Kim and Nam, 2006). Partial complementarity is sufficient, which results in down-regulation of multiple genes by a single miRNA. The off-target effects observed upon siRNA treatment could therefore arise from partial complementarity to untargeted genes and subsequent inhibition of translation (Doench, et al., 2003; Saxena, et al., 2003; Scacheri, et al., 2004; Zeng, et al., 2003). However, since some miRNAs have also been shown to regulate their targets at the mRNA level (Lim, et al., 2005), similar unintended mRNA down regulation could also occur upon siRNA treatment (Jackson, et al., 2003; Lin, et al., 2005).

Among the screens that have been previously carried out, although some of them did not seem to suffer from off-target effects (Kittler, et al., 2004; MacKeigan, et al., 2005; Pelkmans, et al., 2005), whereas others concluded that their major phenotypes were actually due to the down-regulation of unintended genes (Lin, et al., 2005; Ma, et al., 2006). In particular, Lin et al. showed that a partial complementation of only 7 nucleotides was sufficient to induce mRNA down-regulation of an unintended gene (Lin, et al., 2005). These 7 nucleotides correspond to the seed region of miRNA, which is also necessary for miRNA-induced down-regulation (Birmingham, et al., 2006; Jackson, et al., 2006; Lin, et al., 2005).

The difference in off-target occurrence between these various screens could arise from several causes. First, in the screens that do not show off-targets, only a small subset of genes has been tested. It is therefore not unlikely that other phenotypes are due to off-target effects. Furthermore, it is possible that some cellular processes would be more sensitive to off-target effects than others. In the case of lipid metabolism, several miRNAs have been implicated in its regulation (Esau, et al., 2006; Esau, et al., 2004; Krutzfeldt, et al., 2005; Krutzfeldt and Stoffel, 2006). It is possible that several genes implicated in lipid homeostasis

are targets of miRNAs and would therefore be more sensitive to unintended down regulation by siRNA (Jackson, et al., 2006; Stark, et al., 2005). Supporting this hypothesis, a high proportion of genes implicated in lipid metabolism have long 3' untranslated region (UTR), the part of the gene that is preferentially targeted by miRNAs. Nevertheless, some miRNAs still wait to be discovered and other sequences in the 3'UTR could be responsible for the off-targeting. Unfortunately, considering the high number of genes that contain a 7 nt complementarity to a given siRNA in their 3'UTR, such homology cannot be used to identify the potential off-targets (Birmingham, et al., 2006).

Several suggestions have been made to decrease the occurrence of off-target effects. One of them is the use of lower siRNA concentration. It has been suggested that decreasing the siRNA concentration would not impair down-regulation of the targeted gene, but decrease the occurrence of off-target effects (Persengiev, et al., 2004). Even though this might be true for very efficient siRNAs, however the down-regulation of the target may be affected upon use of a less potent siRNA, thereby increasing the occurrence of false negatives in a screen. For this reason, high siRNA concentrations were also used in previous screens (MacKeigan, et al., 2005; Pelkmans, et al., 2005). Furthermore, decreasing the siRNA concentration most of the time did not disrupt the potential off-target phenotypes (Jackson, et al., 2003). Similarly, esiRNAs have been suggested to generate less off-target effects due to the low concentration of any individual sequence (Kittler, et al., 2007). However, although some esiRNAs were very potent in knocking down their targets, some others showed only minor down-regulation, thereby also increasing the occurrence of false negatives in a screen.

Pooling of multiple siRNAs targeted against the same gene has been proposed to increase (http://www.ambion.com/techlib/tn/121/11.html) or decrease (http://www.dharmacon.com/docs/article_pooling.pdf) off-target effects. Therefore another suggestion is to directly compare the effect of several individual sequences targeting the same gene independently in the primary screen. Although variation in efficiency may result in different phenotypes, it again appears as a sensible way to directly select potential real targets. In addition, nowadays, most suppliers have improved their siRNA design to reduce off-target effects. Also further understanding of the RNA interference process and improvement of the siRNA design accordingly may therefore support the generation meaningful screening results in the future. Till date there is no consensus on the best strategy with respect to pooling several siRNAs against one gene or to

screen them independently. Keeping in mind all the available knowledge, I decided to use a low siRNA concentration of 10nM in combination with a very efficient transfection reagent Hiperfect (Qiagen). Additionally I also decided to incorporate both the strategies of pooling and non-pooling of siRNA sequences in this study. The primary screen was performed with two siRNA sequences targeting a single gene pooled together in one well as it would be more expensive to screen them separately in the primary screen with a large number of samples. However, to rule out the false positives due to off-target effects hit validation was performed by using 4 independent siRNA sequences targeting the same gene for all the 132 hits from the primary screen. By this combinatorial approach I aimed to produce a least amount of false positive hits.

RNAi screening experiments using large siRNA libraries targeting genes generate a huge amount of data. Therefore, they require a robust statistical analysis tools in order to identify the most effective siRNAs and to score high confidence hits. There are currently no standard statistical tools for analysis of RNAi screening data unlike for gene expression data analysis. Therefore it is critical to adopt and develop appropriate statistical methods depending on the individual needs. A well-defined and highly sensitive test system requires both quality control and accurate measurements. Within-plate reference controls are typically used for these purposes. Controls help to identify plate-to-plate variability and establish assay background levels. Normalization of raw data removes systematic plate-to-plate variation, making measurements comparable across plates. For that reason, I decided to use two different statistical analysis methods for scoring hits from the primary screen data. The first method was using cellHTS a free software package specifically developed for RNAi screening data analysis based on R and Bioconductor (Boutros, et al., 2006). In this method data normalization was performed with the B Score method before scoring the hits (plate based normalization). The other was a custom developed method where percent of control (POC) normalization was used (control based normalization). A total of 327 targets were identified as primary hits combinedly from both statistical analysis methods. All the primary hits were ranked based on the strength of the phenotype identified in each analysis method and the overlapping hits between both analyses were determined. All the top 80 candidate genes identified to be overlapping from both the analysis methods were chosen for further validation, in addition 26 top non-overlapping candidates each from B Score and POC analysis methods respectively were identified and chosen for validation. This was done

mainly to ensure selection of high confidence hits from the primary screen and also to evaluate the best statistical analysis method. It was intended to be standardized for the future high throughput RNAi screen data analysis at the screening facility of Max Planck Institute for Infection Biology.

POC analysis method was employed to score hits from the hit validation of the 134 primary hits. The final hits were classified in to three categories based on the fold change and the number of siRNAs the phenotype was scored from as described in detail in the section 3.2.5 of results.

Among the strong hits identified leading to significant reduction in the chlamydial infective progeny upon knockdown were, PTEN (phosphatase and tensin homolog) a negative regulator of intracellular levels of phosphatidylinositol-3,4,5-trisphosphate and AKT/PKB Signaling pathway, COPB1 (Coatomer protein complex, subunit beta 1) a protein subunit of the coatomer complex associated with non-clathrin coated vesicles, CDKN1C (Cyclin-dependent kinase inhibitor 1C) a strong inhibitor of several G1 cyclin/Cdk complexes and negative regulator of cell proliferation, FGFR3 (Fibroblast growth factor receptor 3) a member of the fibroblast growth factor receptor family. In the group of strong hits leading to increase in the chlamydial infective progeny upon knockdown were, MAPK14 (Mitogen-activated protein kinase 14) a MAP kinase family member known to be involved in stress related transcription and cell cycle regulation, as well as in genotoxic stress response, DIABLO (Drosophila diablo homolog) a moderator of the caspase inhibition of IAPs, GUK1 (Guanylate kinase 1), GRK5 (G protein-coupled receptor kinase 5) a regulator of the activated forms of G protein-coupled receptors, RAF1 (v-raf-1 murine leukemia viral oncogene homolog 1) a MAP3 kinase which functions downstream of the Ras family of membrane associated GTPases, TNIK (TRAF2 and NCK interacting kinase) known to activate the c-Jun N-terminal kinase (JNK) pathway and regulate actin cytoskeleton by acting as a Rap2 effector, PTK2 (Protein tyrosine kinase 2) a member of the FAK subfamily of protein tyrosine kinases involved in cell growth and intracellular signal transduction pathways, KRAS (Kirsten rat sarcoma viral oncogene homolog) a member of the small GTPase super family implicated in various malignancies, TAX1BP1 (human T-cell leukemia virus type I binding protein 1), RHOA (Ras homolog gene family, member A) a member of the Ras homology family of small GTPases involved in the regulation of cytoskeleton. The hits represent a broad range of

cellular functions and molecular processes emphasizing the diverse interactions between Chlamydia and host cell.

Cellular responses are orchestrated by complex molecular networks. Information contained in primary databases and in the experimental literature relevant to these networks is extensive. Therefore, we used Ingenuity Pathway Analysis software (IPA), a web-based interface, to query molecular interactions, biological functions, for generating a customized pathway analysis. The validated hits were grouped according to proposed molecular and cellular functions. These groups were subjected to a network analysis using IPA to find additional involved molecules. Connections between these molecules were generated using protein-protein interactions, activation, inhibition and phosphorylation.

From our results, it was found that depletion of the lymphotoxin beta receptor (LTBR), Diablo, TNFR18 and PIP3k4b led to increased Ctr infectivity. In line with this, Leukotriene B4 (LTB4) and IL-6, interacting with LTBR and have been described to increase after C.suis infection (Reinhold, et al., 2008). PIP4K2B KD led to increased Ctr growthin our results, which is shown to bind to TNFRSF1A in MCF7 cells (Castellino, et al., 1997). TNFRSF1A has been shown to be reduced after Ctr infection (Paland, et al., 2008). Supporting our results, TNFRSF1A as well as TNFSF9 and TNFRSF18 have been described to lead to increased Cpn growth when inhibited with TNF-alpha (Njau, et al., 2009). Moreover, Diablo interacts with IAPs (Rajalingam, et al., 2007), which are essential for anti-apoptosis in Ctr infected cells (Rajalingam, et al., 2006). Diablo, LTBR, TNFR18 and TNFRSF1A can interact with TRAF2 (Esparza and Arch, 2005; Kuai, et al., 2003; Papin and Subramaniam, 2004), a protein required for TNF-alpha-mediated activation. TRAF2 can interact with the validated hits TAOK3, MAP3K14, TNIK and MAP2K1, leading to activation of JNK (Darnay, et al., 1999; Fu, et al., 1999; Murai and Pasquale, 2003; Taira, et al., 2004). JNK has been described to interact with Ras (Tibbles and Woodgett, 1999) and depletion of Ras and Raf1, part of major signaling pathways led to increased infectivity. Also prohibitin which interacts with Raf1 (Rajalingam and Rudel, 2005), was identified to increase infectivity upon depletion. Interestingly, depletion of MAP2K6, a downstream target of Raf1 led to decreased infectivity. Ras and Raf1 were identified to play a major role in the described networks. The observed increase in Ctr infectivity upon Ras and Raf1 depletion, as well as the decrease in infectivity upon MAP2K6, a member of the Mek group suggests a different role of Ras, Raf1 and Mek during infection. Knockdown of Ras and Raf components of the Ras/Raf/Mek/Erk

pathway seem to significantly benefit the Chlamydia growth as the infectivity from this knockdowns was 2-3 fold more compared to control in at least 3 of the 4 siRNAs tested for each target. We focused here on the role of Ras and Raf1. The remaining quantitative and qualitative data obtained are open to future in depth analyses.

The Ras/Raf/Mek/Erk cascade is a key signaling pathway involved in events such as cell proliferation, cell-cycle and apoptosis (McCubrey, et al., 2007; Steelman, et al., 2004). Also in Chlamydia infections, Erk activation is associated with activation of cPLA2 (Su, et al., 2004), induction of IL8 (Buchholz and Stephens, 2008), and stabilization of the anti-apoptotic Bcl-2 family member Mcl-1 (Rajalingam, et al., 2008). In case of the canonical cascade Mek1/2 activated by Raf1 phosphorylates Erk1/2 on specific tyrosine and threonine residues leading to its activation (McCubrey, et al., 2007). Subsequently Erk1/2 directly phosphorylates a variety of transcription factors including c-Jun and NF kappaB (Nakano, et al., 1998; Steelman, et al., 2004). Our results showed a clear increase in the chlamydial infective progeny upon knockdown of either Ras or Raf in contrast to the published reports by Su et al. (2004). To clarify these seemingly contradictory results and to further elucidate the underlying molecular mechanism involved in the upregulation of chlamydial infectivity in the Ras and Raf1 knockdowns, extensive biochemical as well as microscopical characterisation of these factors was undertaken.

My initial experiments of time course infection of HeLa cells with C. trachomatis to verify if Erk1/2 and cPLA2 phosphorylation showed comparable results which are in concordance to the previously published results (Su, et al., 2004) (Figure 21). Subsequent experiments comparing the Erk and cPLA2 phosphorylation in the presence of chemical inhibitor U0126 used by Su et al. also showed a decreased activation of Erk and cPLA2 and a decrease in the Chlamydia infectivity (Figure 22**Error! Reference source not found.**) what is also in compliance with the previous results. In addition knockdown of Mek1/2 using siRNA also showed decreased Ekr1/2 phosphorylation (Figure 23) and Chlamydia infectivity. Taken together, these results suggest that the phosphorylation of Erk1/2 and subsequent activation of cPLA2 are important for better growth of Chlamydia inside the host cells. Also showed, evidently that the activation of Erk1/2 in Chlamydia infections is dependent on Mek1/2.

However, knockdown of either Ras or Raf1 using siRNA in Chlamydia infections did not led to a decrease in of Erk1/2 and cPLA2 phosphorylation (Figure 24**Error! Reference source not**

found.). Furthermore, I could also observe a prominent increase in the Chlamydia infectivity in the knockdown conditions of both Ras and Raf1 confirming the results of the screen. Thus it is very unlikely that the Ras and Raf1 are involved in the downstream activation of Mek1/2 and Erk1/2 of the Ras/Raf/Mek/Erk pathway in Chlamydia infections. In case of the Mycobacterium tuberculosis infection of monocytes/macrophages, Erk1/2 activation has also been shown to be independent of Ras and Raf1 (Yang, et al., 2007)). However our results from Ras and Raf1 are seemingly in contradiction with the previously published results (Su, et al., 2004). Therefore, I further investigated this apparent discrepancy in my results and the existing knowledge about the activation of this important Signaling pathway in Chlamydia infections. One major point was to elucidate how Ras and Raf1 are uncoupled from the Ras/Raf/Mek/Erk pathway in Chlamydia infection.

The kinase activity of Raf 1 (Moelling, et al., 1984) has been shown to be regulated by phosphorylation of a highly conserved serine residue (Ser259) in the amino-terminal regulatory domain (Morrison and Cutler, 1997). Furthermore phosphorylation of Ser259 is shown to result in Raf1 inactivation as mutation of Ser259 to Ala constitutively activates the kinase activity of Raf1 (Rommel, et al., 1996) and this modification leads to binding of Raf1 to 14-3-3β protein, a negative regulator of Raf1 (Zimmermann and Moelling, 1999). I therefore explored the possibility that the Akt kinase might directly phosphorylate Raf1 at Ser259 (Zimmermann and Moelling, 1999) leading to its inactivation in Chlamydia infection. Subsequently I could show a clear increase in the Raf1 phosphorylation at Ser259 in Chlamydia infected cells using a specific antibody on western blot. I could also confirm AKT as the responsible kinase for this phosphorylation (Figure 25**Error! Reference source not found.**). These results demonstrate the inactivation of Raf1 in normal Chlamydia infection of the host cells to be actively modulated by the pathogen for its benefit. This explains the increase of infectivity observed in the Ras and Raf1 knockdown conditions in the screen.

It is reported that Chlamydia infection induces activation of PI3K /Akt pathway in host cells, which is speculated to be through secretion of effector proteins via the type III secretion apparatus directly or indirectly by oxidative or mechanical stress induced by infection (Verbeke, et al., 2006). PI-3 kinase and Akt pathway acts downstream of Ras and is known to regulate various cellular processes acting either synergistically with (Rodriguez-Viciana, et al., 1997) or in opposition to (Hu, et al., 1996) the Raf pathway. Several pro-apoptotic proteins have been identified as downstream targets of Akt. One of these is the Bcl-2 family

member Bad (Datta, et al., 1997; del Peso, et al., 1997). Phosphorylation of Bad triggers association with 14-3-3 proteins and loss of apoptotic activity in Chlamydia infections (Verbeke, et al., 2006). Akt also phosphorylates the forkhead transcription factor FKHRL-1, and as with Bad, this phosphorylation has been shown to led to its association with 14-3-3 proteins and loss of FKHRL-1 function (Brunet, et al., 1999). Akt is also reported to antagonize Raf1 activity by direct phosphorylation of Ser259 of Raf1 (Zimmermann and Moelling, 1999).

In Chlamydia trachomatis-infected cells, 14-3-3β is known to co-localize to the inclusion via direct interaction with a C. trachomatis encoded inclusion membrane protein IncG (Scidmore and Hackstadt, 2001). This taken together with the fact that Ser259 phosphorylated Raf1 binds to 14-3-3β suggests that this IncG bound 14-3-3β might sequesters Raf1 to the inclusion membrane from the cytoplasm of the host cells. Conforming to this hypothesis, I could clearly demonstrate co-localization of the total Raf1 and p-Raf1 (Ser259) with 14-3-3β at the Chlamydia inclusion membrane (Figure 26). Furthermore, the re-localization of the Raf1 to the inclusion membrane was inhibited by knockdown of 14-3-3β and AKT (Figure 27**Error! Reference source not found.**) indicating that this process to be actively regulated by Chlamydia by Ser259 phosphorylation of Raf1 through AKT. The inactivation of Raf1 by phosphorylation and its recruitment to the inclusion membrane through 14-3-3β indicates that Raf1 in its active state might be evoking a host cell response that might be unfavourable for Chlamydia survival in the host cell.

In summary, I was able to successfully establish an assay to monitor the infection and infectivity of C. trachomatis in human cells. This in combination with the siRNA screening approach, I was able to identify several important host cell factors involved in the Chlamydia host cell interactions. Furthermore, through a mechanistic study I could clearly demonstrate that the Ras and Raf1 are not required for activation of Mek1/2 and downstream effectors and the uncoupling of Ras and Raf1 from Erk activation in Chlamydia infections. Additionally I have shown that the Raf1 is inactivated by phosphorylation through AKT and recruited to the Chlamydia inclusion in AKT and 14-3-3β dependent manner. However, the functional significance of Raf1 inactivation is still unclear at this stage. It can be speculated that Chlamydia specifically inactivates and sequesters the Raf1 to create conducive environment for its survival and replication by inhibiting downstream functions that Raf1 is involved in inducing. Thus, it is interesting to further investigate the functional relevance of Raf1

inhibition by Chlamydia on its survival, which might offer new possibilities to tackle the Chlamydia infections. Therefore, I have demonstrated in this study that the application of global RNAi screening approach can lead to identification of thus far unknown processes enabling a comprehensive understanding of the host-pathogen interactions. Besides, I are sure that further functional characterization of all or most of the important identified factors might unravel so far unknown and important host and Chlamydia interactions, which might help in broader understanding of the diseases caused by this pathogen to evolve better treatment strategies.

5 Materials and methods

5.1 Materials

5.1.1 Bacterial Isolates

Chlamydia trachomatis LGV L2: Lymphatic isolates (ATCC VR-902B)

5.1.2 Cell lines

HeLa human epithelial cells from cervical adenocarcinoma (ATCC CCL-2) were obtained from the American Type Cell Collection (ATCC).
HeLa 229 human epithelial cells from cervical adenocarcinoma (ATCC CCL-2.1) were obtained from the American Type Cell Collection (ATCC).

5.1.3 Cell culture media

5.1.3.1 Growth medium (GM)

Growth medium was used for the maintenance of HeLa cells and consisted of RPMI 1640 with 2 mM L-glutamine and 25 mM HEPES (GibCo) supplemented with 10% FBS, heat inactivated (Biochrome).

5.1.3.2 Infection medium (IM)

Infection medium was used for performing Chlamydia infection experiments and consisted of RPMI 1640 with 2 mM L-glutamine and 25 mM HEPES (GibCo) supplemented with 5% FBS, heat inactivated (Biochrome).

5.1.4 Buffers and solutions

5.1.4.1 Blocking Buffer for Immunoflourescence

BSA (0.2%) in PBS

5.1.4.2 PBS (10X)

2 g/l KCl

2 g/l KH2 PO4

80 g/l NaCl

11.5 g/l Na2 HPO4

pH 7.4

5.1.4.3 SDS-electrophoresis buffer (10x)

1.92 M Glycine

250 mM Tris/HCl

1% SDS

5.1.4.4 SDS loading buffer (4x, reducing)

125 mM Tris/HCl pH 6.8

6% SDS

40% Glycerol

10% □-Mercaptoethanol

2-10 mg Bromophenol blue

5.1.4.5 SDS Stacking gel buffer (4x)

6.05 g Tris/HCl

0.4 g SDS

Add 100 ml H20

Adjust to pH 6.8 with HCl

5.1.4.6 SDS resolving gel buffer (4x)

91 g Tris/HCl

2.0 g SDS

Add 500 ml H2O

Adjust pH to 8.8 (with HCl)

5.1.4.7 SPG buffer

75 g Sucrose

0.52 g KH2PO4

1.22 g Na2HPO4

0.72 g L-glutamic acid

Fill up to 1 l with distilled water.

Adjust pH to 7.4

Sterile filter and store at 4°C

5.1.4.8 TBS-buffer

20 mM Tris/HCl

140 mM NaCl

pH 7.5

For TBS-T, add 0.05% Tween 20

5.1.4.9 Blocking buffer (Western blot)

TBS-T

3% milk powder

5.1.4.10 TBE buffer

89.15 mM Tris/HCl

88.95 mM Boric acid

2 mM EDTA

5.1.4.11 Western blot stripping buffer

62.5 mM Tris/ HCl pH 6.7

100 mM □-Mercaptoethanol

2% SDS

5.1.4.12 Wet blot transfer buffer

6 g Tris

28.8 g Glycine

2 g SDS

20% Methanol

Add up to 2 l H20

5.1.4.13 Protein Marker

PageRuler™ Plus Prestained Protein Ladder (Fermantas, # SM1811)

5.1.4.14 Mowiol embedding medium

Mowiol 4-88 (2.4 g)	20% w/v
Glycerol (6 g)	50%
H 20 (6 ml)	
Tris/HCl pH 8.5(12 ml 0.2 M) 100 mM	

Let swell over night;

Heat to 50°C to dissolve;

Optional: 10% w/v DABCO to slow down bleaching

5.1.5 Fine chemicals

Mek 1/2 Inhibitor U0126 (Sigma, # U120)

Hoechst 33342 (Sigma, # B2261)

5.1.6 Kits used in this study

5.1.6.1 Sub-cellular fractionation kit

For sub cellular fractionation of the cells, the ProteoExtract Subcellular Proteome Extraction kit (Calbiochem, # 539790) was used. The fractionation was performed as per the manufactures instructions.

5.1.7 Primary antibodies

Table 2: List of the primary antibodies employed in this study

Antigen	Species	Dilution for IF	Dilution for WB	Company
anti-Chlamydia MOMP	Mouse	1:10000	-	University of Washington
anti - HSP 60	Mouse	-	1:5000	Alexis, # 804-072-R100
Lamin a/c	Mouse	-	1:200	Chemicon, # MAB3211???
□-Actin	Mouse	-	1:10000	Sigma, # A1978
anti-c-Raf	Rabbit	1:100	1:1000	Cell Signaling, # 9422

anti-c-Raf	Rabbit	1:100	1:500	Santa Cruz, # sc-7198
anti-Ras	Rabbit	-	1:1000	Cell Signaling, # 3965
anti-Phospho-cPLA2	Rabbit	-	1:1000	Cell Signaling, # 2831
anti-cPLA2	Rabbit	-	1:1000	Cell Signaling, # 2832
anti-Phospho-p44/42 MAPK (ERK1/2)	Mouse	-	1:2000	Cell Signaling, # 9106
anti-p44MAP Kinase (Erk1)	Rabbit	-	1:1000	Cell Signaling, # 4372
anti-phospho-cRaf(Ser259)	Rabbit	1:100	1:1000	Cell Signaling, # 9421
anti-phospho-cRaf(Ser259)	Rabbit	1:100	1:500	Santa Cruz, # sc-101791
anti – 14-3-3β	Goat	1:100	-	Santa Cruz, # sc-17288
anti – Cytokeratin 8	Mouse	-	1:500	Santa Cruz, # sc-58736
anti –Lamp1	Rabbit	-	1:1000	Cell Signaling, # 3243
anti-MEK1/2	Rabbit	-	1:1000	Cell Signaling, # 9126
anti-Akt	Rabbit	-	1:1000	Cell Signaling, # 9272
anti-Calpain	Rabbit	-	1:1000	Cell Signaling, # 2539

5.1.8 Secondary antibodies

Tabelle 3: List of the secondary antibodies employed in this study

Antigen	Species	Dilution for IF	Dilution for WB	Company
anti-Mouse-Cy3	Goat	1:100	-	Jackson ImmunoResearch, # 115-165-146
anti – Goat-Cy2	Donkey	1:100	-	Dianove, # 705-225-147
anti – Rabbit-Cy5	Goat	1:100	-	Dianove, # 111-175-144
anti – Rabbit-HRP	Donkey	-	1:2000	Amersham, # NA934
anti – Mouse-HRP	Sheep	-	1:2000	Amersham, # NA931

5.1.9 siRNA

siRNAs were purchased from Qiagen, if necessary siRNA were validated at the Max Planck Institute for Infection Biology for their ability to knock down mRNA expression of target genes by more than 70% in comparison with control cells transfected with siRNA targeting luciferase.

Tabelle 4: List of siRNAs used in this work (except the kinase and apoptosis libraries)

Gene	ID Number	RefSeq Number
ABI1	10006	AF540955.1
ABI2	10152	U23435.1
ABP-280	2316	X53416.1
ACTN1	87	NM_001102.2
AIF	9131	AF100928.1
AKT1	207	NM_005163
ALDH1A2	8854	NM_003888
ALOX5	240	NM_000698.1
ANP32A	8125	NM_006305
AP1B1	162	NM_001127
AP1M1	8907	NM_032493

AP1S1	1174	NM_001283
AP1S2	8905	NM_003916
Apaf-1	317	AF013263.1
APG5L	9474	NM_004849.1
APG7L	10533	NM_006395.1
APO-1	355	X63717.1
Apo-2	8743	U57059.1
AQP5	362	NM_001651.1
ARC34	10109	AF006085.1
ARD-1	8260	X77588.1
ARF1	375	NM_001658
ARF3	377	NM_001659
ARF4	378	NM_001660
ARF5	381	NM_001662
ARF6	382	NM_001663
Arp3	10096	AF006083.1
Asc2	23054	AF177388
ATM	472	NM_000051.2
ATP5B	506	X03559
ATP5H	10476	NM_006356.1
ATPsAC	498	X59066.1
ATPsEC	514	NM_006886
ATPsGC	509	NM_005174
ATR	545	NM_001184.2
Aven	57099	AF283508
BAD	572	AF031523.1
BAG1	573	NM_004323
BAK1	578	NM_001188
BARD1	580	NM_000465
BAT1	7919	Z37166.1
BBC3	27113	NM_014417

Bcl 2	596	NM_000633.1
Bcl2A1	597	NM_004049.2
BCL2L10	10017	NM_020396
BCL3	602	NM_005178
BCL6	604	NM_001706
BCR	613	NM_004327
BECN1	8678	NM_003766.2
Bid	637	NM_197967
BIK	638	U34584
BimL	10018	AF032458
BIRC6	57448	NM_016252
Bmf	90427	AY029254
BNIP1	662	U15172
BNIP3	664	NM_004052
BNIP3L	665	AF079221
BOK	666	AF089746
BRAF	673	NM_004333.1 (004333.2)
BRCA1	672	NM_007294.1
BWR1C	7262	AF035444 (AF019953)
CALR	811	BC002500.2
CARD8	22900	AF322184
CASP1	834	NM_001223.2
CASP10	843	NM_001230.2
CASP3	836	NM_004346.2
CASP4	837	BC017839.1
CASP5	838	Nm_004347.1
CASP6	839	NM_001226.2
CASP7	840	NM_001227.2
CASP8	841	NM_033358.1
Casp9	842	NM_001229
CAV1	857	NM_001753.3

CBX1	10951	NM_006807.2
c-CBL	867	NM_005188
CCND1	595	NM_053056.1
CCNE1	898	NM_001238
CCT2	10576	AF026293.1
CD30	943	M83554.1
CD36	948	NM_000072.1
CD44	960	U40373.1
CD46	4179	NM_002389.3
cdk2	1017	X62071.1
CDK4	1019	NM_000075
CDKN1A	1026	NM_000389.2
CDKN1B	1027	NM_004064
CDKN1C	1028	NM_000076
c-erb-B-2	2064	X03363.1
CFL1	1072	NM_005507.1
CHEK1	1111	NM_001274
CHEK2	11200	NM_007194
CHUK	1147	NM_001278.2
c-IAP1	329	L49431.1
c-IAP2	330	L49432.1
CLTA	1211	NM_001833
CLTC	1213	NM_004859.1
COPB	1315	NM_016451
CORO1A	11151	NM_007074.1
CPSF5	11051	NM_007006.1
CREB1	1385	NM_134442.2
CRHSP-24	23589	AF115345.1
CRK-II	1398	D10656.1
CSA2	3550	AF182645.1
CSK	1445	NM_004383.1

CTNNA1	1495	NM_001903.1
DAXX	1616	NM_001350.2
DAZAP1	26528	NM_018959.2
DcR1	8794	AF012536.1
DcR2	8793	AF023849.1
DcR3	8771	AF104419.1
DDX1	1653	NM_004939
DHFR	1719	NM_000791
DJ-1	11315	XM_001707.4
DNER	92737	BC035009
DNM2	1785	NM_004945
DNMT1	1786	NM_001379
DR3	8718	AF026070.1
DR5	8795	AB014710.1
DrpI	10059	NM_012062.2
DSP	1832	NM_004415
DUSP5	1847	NM_004419
E2F1	1869	NM_005225.1
E2F4	1874	NM_001950.3
EEF1A2	1917	NM_001958
EEF1B2	1933	NM_001959.2
EEF1D	1936	NM_001960.2
EGFRBP-GRB2	2885	M96995.1
EIF2AK2	5610	U50648 NM_002759
EIF3H	8667	NM_003756
EIF4E	1977	NM_001968
EIF5A	1984	NM_001970
ELAVL1	1994	NM_001419.1
ELK1	2002	NM_005229
EMS1	2017	M98343.1
EPHA2	1969	M59371

EZR	7430	BC013903.1
FADD	8772	U24231
FAK	5747	L13616
FGFBP1	9982	NM_005130
FLJ22625	79770	BC001615
FLOT1	10211	NM_005803.2
FRAP1	2475	NM_004958.2
FTH1	2495	NM_002032.1
FTL	2512	NM_000146.2
FUBP1	8880	NM_003902.2
FYN	2534	NM_002037
G3BP	10146	NM_005754
G3BP2	9908	AF051311
GAB1	2549	U43885.1
GABARAP	11337	NM_007278.1
GADD45A	1647	NM_001924.2
GADD45B	4616	NM_015675
GADD45G	10912	NM_006705
GLUD1	2746	NM_005271
GMPS	8833	NM_003875.1
GOLGB1	2804	NM_004487
GRP58	2923	NM_005313
GST-Pi	2950	X06547.1
HADH2	3028	NM_004493.1
HADHA	3030	NM_000182
hAPG12	9140	AB017507.1
hB23	4869	X16934
HBE1	3046	NM_005330
HBG1	3047	BC020719
HCA56	1939	AF220417
HDGF	3068	NM_004494.1

HEP06676	54888	AK000310.1
hnRNP G	27316	NM_002139.1 (AY464692)
hnRNP R	10236	AF000364.1
HNRNPD	3184	D55671.1
HNRNPH1	3187	L22009.1
HNRPA0	10949	NM_006805.2
HNRPA1	3178	XM_028653.1
HNRPA2B1	3181	XM_004930.3
HNRPAB	3182	NM_004499.2
HNRPC	3183	NM_004500.2
HNRPK	3190	NM_002140.2
HNRPL	3191	NM_001533.1
HNRPM	4670	XM_045150.1
HNRPX	5093	U24223.1
HRAS	3265	NM_005343
HSABL	25	X16416.1
HSBTF3B	689	X53281.1
HSEGFPRE	1956	X00588
HSP60	3329	M34664.1
HSPA9B	3313	NM_004134.3
HSPH1	10808	NM_006644
HSPHAPI2A	10541	Y07569.1
HSRAP1A	5906	X12533
HSRHO2	397	X69549.1
HSSCHAD	3033	X96752.1
HSSYKPTK	6850	Z29630
HSTRAPA	959	X68550.1
HSTRR	7037	X01060.1
HSU2AR	6627	X13482.1
HUMASF	6426	M72709
HUMBAXA	581	L22473.1

HUMCDPKTIA	5592	D45864
HUMDLDH	1738	J03620
HUMHFP	3185	L28010.1
HUMKER1A	5595	M84490.1
HUMLAMC	4000	M13451
HUMPGES	5742	M59979.1
HUMPSP45	5705	D44467.1
HUMTRL	291	J02966
HUMVINC	7414	M33308
Ich-1	835	U13021.1
IDH2	3418	NM_002168
IDH3A	3419	NM_00553
IER3	8870	S81914
IFNB1	3456	NM_002176.1
IKBKB	3551	AF080158
IKBKE	9641	AF241789.1
IKBKG	8517	AF261086
IL8	3576	BC013615
IL8RB	3579	NM_001557
IMP	3615	J04208
IMP-1	10642	NM_006546.2
IMP-3	10643	AF117108.1
IPAF	58484	AY035391
IQGAP1	8826	NM_003870.2
IRAK4	51135	NM_016123
ITGA5	3678	NM_002205.1
ITGB1	3688	NM_002211.2
ITGB4	3691	NM_000213
JIP2	23542	AF136382.1
JKTBP	9987	D89092.1
JNK1	5599	L26318.1

JNK2	5601	L31951.1
JUN	3725	NM_002228.2
JUND	3727	NM_005354
K12H4.8	23405	AJ132261.2
KHSRP	8570	NM_003685.1
KIF1C	10749	U91329
KIF20A	10112	NM_005733
KIF3A	11127	BC045542
KIF3B	9371	NM_004798
KIF5B	3799	NM_004521
KIF9	64147	NM_182902
KIFAP3	22920	NM_014970
KISS1	3814	NM_002256
KIT1P	889	AF310133
KRAS	3845	NM_004985
LAMB2	84823	M94362.1
LAMR1	3921	NM_002295.2
LASP1	3927	NM_006148
LCK	3932	NM_005356
LDHA	3939	NM_005566.1
LMNB1	4001	NM_005573
MACF1	23499	NM_012090
MAP1LC3A	84557	NM_032514.2
MAP1LC3B	81631	NM_022818.2
MAP2K1	5604	NM_002755
MAP2K2	5605	NM_030662
MAP2K4	6416	NM_003010.2
MAP2K7	5609	NM_145185.1
MAP3K14	9020	NM_003954.1
MAP3K2	10746	NM_006609.2
MAP3K5	4217	NM_005923.3

MAP3K7	6885	AB009357.1
MAPK1	5594	M84489
MAPK14	1432	NM_001315.1
MAX	4149	NM_002382.3
MCL1	4170	L08246 (AF118124)
MDM2	4193	NM_002392.1
MEFV	4210	NM_000243
MEKK1	4214	AF042838.1
MET	4233	NM_000245.1
MKI67	4288	NM_002417.2
MSN	4478	BC017293
MSR1	4481	NM_138716,
MTX1	4580	NM_002455
MYC	4609	NM_002467.2
MyD88	4615	U70451
NACA	4666	NM_005594.1
NAIP	4671	U19251
NALP1	22861	AF310105
NALP10	338322	AY154465
NALP11	204801	AY154466
NALP12	91662	AY154467
NALP13	126204	AY154468
NALP14	338323	AY154469
NALP2/NALP7	55655	AF310106
NALP3L	114548	AF468522
NALP4	147945	AF442488
NALP5	126206	AY154460
NALP6	171389	AY154461
NALP8	126205	AY154463
NALP9	338321	AY154464
NAP1RP	4673	NM_004537.3 (BT007023)

NCK1	4690	NM_006153
NCL	4691	NM_005381.1
NFKB1	4790	NM_003998.1
NFKB2	4791	NM_002502
NKEFA	5052	L19184
NMHC	4627	M31013.1
NMP200	27339	AJ131186.1
nmt55	4841	U89867.1
NOS1	4842	NM_000620
NOS2A	4843	NM_000625
NOS3	4846	NM_000603
NRAMP2	4891	NM_000617
NRAS	4893	NM_002524
P1-Cdc21	4173	X74794.1
p42	8666	AF020833
p73	22062	Y19235
PABPC4	8761	NM_003819.2
PAFAH1B3	5050	NM_002573.2
PAGE-5	90737	NM_130467
Pak1	5058	U24152.1
Pak2	5062	U24153
PDGF	5159	J03278.1
PFN1	5216	NM_005022
PHB	5245	NM_002634
PHLDA1	22822	Z50194 (NM_007350)
PLA2G4A	5321	NM_024420
PLCG1	5335	M34667.1
PLEC1	5339	NM_000445
por	7417	L08666.1
PR264	6427	X62447.1
PRDX2	7001	NM_005809

PRDX3	10935	NM_006793.2
PRKCA	5578	NM_002737
PRKCB1	5579	NM_002738
PRKCD	5580	NM_006254
PRKCE	5581	NM_005400
PRKCG	5582	BC047876
PRKCH	5583	NM_006255
PRKCI	5584	NM_002740
PRKCM	5587	NM_002742
PRKCN	23683	NM_005813
PRKCQ	5588	NM_006257
PRKCZ	5590	NM_002744
PSMA3	5684	NM_002788
PSMA7	5688	BC004427
PSMB1	5689	BC020807
PSMC3	5702	NM_002804
PSMC4	5704	NM_006503
PSMD7	5713	NM_002811.2
PSPC1	55269	AK001817.1
PTBP1	5725	NM_002819.3
PTEN	5728	NM_000314
PTGS2	5743	NM_000963.1
Rab 11B	9230	NM_004218
Rab 1B	81876	NM_030981
Rab 1C/Rab35	11021	NM_006861
Rab 33A	9363	NM_004794
Rab 6B	51560	NM_016577
Rab 6C	84084	NM_032144
RAB10	10890	NM_016131
RAB11A	8766	NM_004663.2
RAB1A	5861	NM_004161

RAB2	5862	NM_002865
RAB30	27314	NM_014488
RAB33B	83452	NM_031296
RAB4A	5867	BC004309.1
RAB5A	5868	NM_004162
RAB6A	5870	NM_002869.2
RABIF	5877	NM_002871
RAC2	5879	M29870
RAC2	5880	NM_002872.2
RAD21	5885	X98294
RAD23B	5887	NM_002874
RAF1	5894	NM_002880.1
RALY	22913	AF148457.1
RB1	5925	NM_000321.1
RBBP4	5928	X71810.1
RBM14	10432	NM_006328
RCC2	55920	AB040903
RDX	5962	NM_002906
RELA	5970	L19067
RIPK2	8767	AF027706
RPLP0	6175	NM_001002.2
RPS18	6222	NM_022551
RPS6KB1	6198	NM_003161.1
SARA1	56681	NM_020150
SEMG1	6406	NM_003007.1
SEPT6	23157	NM_015129
Serpin	5272	BC002538
SF1-Bo	7536	Y08766.1
SFN	2810	NM_006142.2
SFPQ	6421	NM_005066.1
SFRS9	8683	NM_003769.1

SHC1	6464	NM_003029.2 (003029.3)
SH-PTP2	5781	L03535
SIRT2	22933	NM_012237.2
SKP1A	6500	NM_170679.1
SLC3A2	6520	NM_002394.2
SLC7A11	23657	NM_014331.2
SMAC	56616	NM_019887.2
SMARCE1	6605	NM_003079.3
SMC1L1	8243	NM_006306
SMPD1	6609	NM_000543
SMPD2	6610	NM_003080
SNW1	22938	U51432
SOS1	6654	L13857.1
SPTAN1	6709	NM_003127.1
SRC	6714	NM_005417.2
SSNA1	8636	BC015827
STMN1	3925	NM_005563
SURVIVIN	332	U75285.1
SVIL	6840	NM_003174
TAJ-alpha	55504	AF167555.1
TBK1	29110	NM_013254.2
Tensin3	64759	AF417489
TES	26136	NM_015641
TFR2	7036	AF067864.1
TGFBR1	7046	NM_004612
TGFBR2	7048	NM_003242
TIA-1	7072	M77142.1 (NM_022037)
TIAM1	7074	NM_003253
TIP47	10226	NM_005817.2
TJP1	7082	NM_003257.2
TLN1	7094	AF078828

TLR1	7096	U88540.1
TLR2	7097	U88878.1
TLR3	7098	NM_003265.2
TLR5	7100	AB060695.1
TLR7	51284	NM_016562.2
TLR8	51311	AF246971.1
TLR9	54106	AF245704.1
TNFa	7124	AF043342.1
TNFRSF1A	7132	NM_001065.2
TNFRSF1B	7133	NM_001066.2
TNFRSF21	27242	NM_014452.3
TNFRSF5	958	NM_001250.3
TNFRSF7	939	NM_001242.3
TNFSF18	8995	AF125303.1
TNS1	7145	AF225896
TNS2	23371	AF417490
TOMM20	9804	NM_014765.1
TOMM40	10452	NM_006114.1
TOMM70A	9868	NM_014820.1
TP53	7157	NM_000546.2
TRA1	7184	NM_003299.1
TRADD	8717	NM_003789.2
TRAF1	7185	NM_005658.2
TRAF2	7186	NM_145718.1
TRAF3	7187	NM_003300.2
TRAF4	9618	NM_004295
TRAF5	7188	NM_004619
TRAF6	7189	U78798
TRIP9	4793	L40407
TSG101	7251	NM_006292.2
TUFM	7284	NM_003321

TXNL	9352	NM_004786
U2AF2	11338	NM_007279.1
ULK1	8408	AF045458.1
USF1	7391	NM_007122.2
USF2	7392	NM_003367.1
USP10	9100	XM_033922.2
VASP	7408	NM_003370.1
VAV1	7409	NM_005428
VDAC3	7419	NM_005662.3
VDP	8615	NM_003715
VEGFA	7422	X62568.1
VEGI	9966	AF039390.1
VIM	7431	NM_003380
WT1	7490	NM_000378.2
XIAP	331	U45880.1
YES1	7525	NM_005433

5.2 Methods

5.2.1 Cell culture

Cell lines were cultured in appropriate growth medium at 37°C in a cell culture incubator under humidified atmosphere containing 5% CO2. Presence of Mycoplasma spp. was excluded using the VenorGeM PCR assay. Before passaging, adherent cells (epithelial and endothelial cells) were washed once with PBS. 1.5 ml Trypsin (0.05%)-EDTA (0.53 mM) was added per 75 cm3 culture flask and cells were incubated at 37°C until they detached. Cells were resuspended in growth medium and transferred into fresh culture flasks in a 1:4 to 1:6 dilutions. To maintain cells in culture and avoid complete confluence, cells were split every two to three days.

5.2.2 Preparation of Chlamydia stocks

For the preparation of C. trachomatis stocks, the bacteria were propagated in HeLa cells grown in 150cm2 cell culture flasks with 24 ml of infection medium. 48 h after infection, the cells were detached using 3 mm glass beads. The cell suspensions were centrifuged at 500 × g and 4°C for 10 min (Hermle Labortechnik, Wehingen, Germany). The pelleted cells were then combined and ruptured using glass beads to ensure the breakage of cells. The lysates were centrifuged as before to sediment nuclei and cell debris. The supernatant was removed and recentrifuged at 20,000 × g for 40 min at 4°C in a SS-34 rotor (Sourvall Instruments, CT, USA) to pellet Chlamydia. The harvested bacteria were resuspended in 15 ml SPG buffer using a 21-22 gauge injection needle. Chlamydia suspensions were then aliquoted and frozen at -75°C and freshly thawed before each experiment.

5.2.3 Assessment of infectivity titer

Ten-fold serial dilutions of freshly thawed C. trachomatis stock EBs were prepared in infection medium and 250 µl of each dilution was inoculated onto a monolayer of HeLa cells cultured in 12-well plates. The bacteria were statically allowed to adsorb to cells for 2 h at 5% CO2 and 37°C. After the adsorption period, cells were washed once with infection medium and then supplemented with 1 ml infection medium per well and incubated again under similar conditions. One day later, the cells were fixed, permeabilized and stained with the Chlamydia genus specific antibody (**Error! Reference source not found.**). The stained inclusions were counted at specific magnifications and the area of the microscopic field used with a micrometer slide (A) was estimated. The total area of the monolayer was estimated as well (B). Fields in the monolayer C= B/A. The number of IFU/ml can be calculated as follows:

$$\frac{\text{No of IFU counted}}{\text{No of Fields examined}} \times C \times DF \times V$$

DF- dilution factor of EB preparation

V- Factor determined by the volume of the inoculum

5.2.4 Infection with Chlamydia

HeLa cells were infected with C. trachomatis at an MOI of 0.5 to 3 in infection medium and grown at 35°C and 5% CO2. Two hour post-infection the medium was replaced by fresh infection medium and incubated until fixed or used for lysis and reinfection. Infections in 96 well plates were performed manually using an automated multichannel pipette from BioHit.

5.2.5 Infectivity Assays

5.2.5.1 Infectivity Assay in 96 well plate

HeLa cells were infected as described before in 3.2.4. Two days post-infection, cells were lysed by adding Nonidet P40(NP40) at a final concentration of 0.06% for 15 min at room temperature, contents were mixed by pipeting up and down 5 times. Plates were subjected to shaking immediately after addition of NP40 and mixing after 15 minutes of incubation. Lysates were then diluted to 1:100 in infection medium before transfer to fresh untreated HeLa cells. After incubation for 24 h at 35°C and 5% CO2, the cells were fixed in ice cold methanol overnight at 4°C, subsequently cells were stained using indirect immunofluorescence protocol described below.

5.2.5.2 Infectivity Assay in 6 well plates

HeLa cells infected with C. trachomatis for 48 hours were scraped off the 6 well plates using a rubber policeman. The cells were collected in 15 ml falcon tube containing sterile glass beads and lysed by vortexing at 2500rpm for 3 minutes. Lysates were then diluted to 1:100 in infection medium before transfer to fresh untreated HeLa cells. After incubation for 24 h at 35°C and 5% CO2, the cells were fixed in ice cold methanol overnight at 4°C, subsequently cells were stained using indirect immunofluorescence protocol described below.

5.2.6 Labeling using indirect fluorescent method

The 96 well screen plates fixed with ice cold methanol were washed 2 times with PBS and then blocked using 0.2% BSA in PBS for 30 min at room temperature. Primary mouse anti C. trachomatis-MOMP KK12 (University of Washington) was added at 1:10,000 dilution in blocking solution for 1 h at room temperature, washed 2 times with PBS and Cy3-labeled goat anti-mouse IgG (Jackson ImmunoResearch) at 1:100 dilution in blocking solution was added for another hour. Host cell nuclei were stained with Hoechst 33342 (Sigma) diluted 1:2000 in blocking solution of the secondary antibody.

5.2.7 Double labeling of cRaf/p-cRaf and14-3-3β markers and confocal microscopy

Infected host cells grown on coverslips were washed 2 times with PBS and then fixed with ice cold methanol overnight at 4°C. Cells were washed 2 times with PBS after discarding the methanol followed by blocking using 0.2% BSA in PBS for 30 min at room temperature. Next, the cells were incubated for 1 h at room temperature with mixtures of either cRaf and 14-3-3β or p-cRaf and 14-3-3β primary antibodies in 100 µl of 0.2% BSA in PBS and then with the secondary fluorochrome-conjugated antibodies diluted in 0.2% BSA-PBS. Between incubation steps, cells were washed at least 3 times with PBS. Finally, the cover slips were washed and mounted using Moviol mounting medium on glass microscopic slides. Mounted specimens hardened at 4°C overnight and sealed with nail polish. The fluorochromes were visualized using filter set responsible for emission of 488 nm (FITC, green color) and 518 nm (rhodamine, red color) and a series of images with Z stacks were acquired using the laser scanning confocal microscope. The stacks were then analyzed using the Imaris Software. Finally the images were processed using Photoshop CS3 (Adobe Systems).

5.2.8 Treatment with Mek1/2 inhibitor U0126

For the experiment with Mek1/2 inhibitor U0126, 1×105 cells/well were seeded in 12 well plates 1 day before infection. Cells were infected with MOI 3 of C. trachomatis in 500 µl infection medium for 2 hours. After two hours the medium was replaced with 1 ml of fresh

infection medium containing either 10 or 100 μM U0126 inhibitor throughout the infection duration.

5.2.9 siRNA transfection

Transfection in 96 well plates was performed using the BioRobot® 8000 system (Qiagen) as follows: 1.5x103 HeLa cells/well were seeded in 96 well plates one day before transfection. For each well to be transfected 0.5 μl from 2 μM siRNA stock was re-suspended in 15 μl of RPMI without serum and incubated for 10 minutes, to this 10 μl of 1:20 diluted Hiperfect (Qiagen, Hilden) reagent was added and mixture was incubated for further 10 min. After incubation 25 μl/well of growth medium was added to the complex. Medium was removed from cultured cells and replaced with 50 μl/well fresh growth medium to this 50 μl/well of the transfection mixture prepared earlier was added leading to final siRNA concentration of 10 nM. Cells were then incubated in 37°C and 5% CO2 incubator for 72h, which was determined to be the optimal time period for effective knock down of most of the target genes.

For functional analysis experiments by Western blot and confocal microscopy: 1×105 cells/well were seeded in 12-well plates 24 h prior to transfection and transfected using Hiperfect (Qiagen) according to the manufacturer's guidelines. In brief, 150 ng of specific siRNA was added to RPMI without serum and incubated with 6 μl Hiperfect transfection reagent in a total volume of 100 μl. After 10–15 min the liposome/RNA mixture was added to the cells with 1 ml cell culture medium leading to final siRNA concentration of 10 nM. After 1 day, cells were trypsinized and seeded into new cell culture plates depending on the experiments. Three days after transfection, the cells were infected and incubated as indicated above.

5.2.10 Automated microscopy and image analysis

chlamydial number, inclusion size and number of cells were analyzed in an automated microscope (Olympus Biosystems). Four pictures were taken from each well with a UV emission using blue filter and green emission with Cy3 filter at the same position. The images were analyzed for the number of cells and the number and size of chlamydial

inclusions by automatically identifying and counting using ScanR Analysis Software (Olympus Biosystems).

5.2.11 SDS-PAGE

SDS-PAGE was carried out using the vertical Biorad Mini-Protean II electrophoresis system. A stock solution of 30% acrylamide plus 0.8% bisacrylamide was used to prepare a 6 cm separating gel of 12% acrylamide and 2 cm stacking gel of 4.3% acrylamide. For polymerization of the gel, 0.1% TEMED and 0.5% APS were used.
After brief centrifugation of the samples, 10 µl of each sample and 5 µl of prestained protein ladder were resolved on the 12% polyacrylamide gel at 75 V until the dye had run out of the stacking gel. The electrophoresis was then continued at 150 V for 50-60 min. The separated proteins were next detected and analyzed by Western blot technique.

5.2.12 Western blot

5.2.12.1 Preparation of cell lysates for Immunoblotting

HeLa cells either with or without transfection depending on the experiment were grown in six-well plates, Infected with C. trachomatis L2 as described previously were washed with PBS. To this 200 µl of 1x SDS-Sample buffer (3% 2-mercaptoenthanol, 20% glycerin, 0.05% bromphenol blue, 3% SDS) was added. The lysate was collected in eppendorf tubes and heated in a boiling water bath or a heating block for 10. Samples were stored at – 20°C until used for further analysis

5.2.12.2 Protein transfer

Proteins separated by SDS-PAGE were transferred electrophoretically to Immobilon-P PVDF membranes using wet (tank) method of blotting in a Biorad electroblotting system (Biorad Mini Trans-Blot Electrophoretic Transfer Cell) according to (Towbin, et al., 1979). The membrane and 2 Whatman 3MM papers were trimmed nearly to the size of the separating gel. The Whatman papers and fiber pads were soaked in transfer buffer, while the PVDF membrane was soaked in methanol for 5 min for activation, washed with distilled water and soaked in transfer buffer. For protein transfer, polyacrylamide gel-membrane sandwich was arranged as follows: the gel was placed on a wetted sheet of Whatman paper already placed

on a well soaked fiber pad on the transfer cassette. The membrane was then placed on the gel, followed by sequential placing of another sheet of Whatman paper and a fiber pad over the membrane. Formation of air bubbles was avoided. Next, the transfer cassette was closed and inserted into the buffer tank containing a small stiring bar and a cooling unit with frozen distilled water. Finally, the transfer apparatus was placed on a magnetic stirrer, electrodes were attached and transfer was carried out for 3 h at 250 mA and 4°C or overnight at 100 mA and 4°C.

5.2.12.3 Immunoblotting

Once protein transfer was completed, the membrane was transferred to a small container with 3% milk powder in TBS-T(blocking buffer) and incubated with shaking on a rocking device for 30 min at room temperature to block any remaining free-binding sites on the membrane. The membrane was then incubated with shaking for 1 h at room temperature or overnight at 4°C with primary antibodies diluted in blocking buffer. After 3 washings for 10 min with TBS-T the bound primary antibodies were incubated with corresponding secondary antibodies conjugated to horseradish peroxidase (Amersham Biosciences and Jackson ImmunoResearch Laboratories) at appropriate dilution in blocking buffer. The membrane was washed 3 times for 10 min with TBS-T. The presence of immunoreactive proteins was detected on an X-ray Kodak film directly or using the AIDA Image Analyzer after soaking the membrane for 1 min in the chemiluminescence solutions 1 and 2 mixed 1:1 (ECL reagent, Amersham Biosciences). Band intensity was calculated using the AIDA Image Analyzer Version 4.03 software. If required the blots were stripped and probed with other antibodies. For stripping, the PVDF membranes were activated by incubating them with methanol for a minute and then washed with TBST for 5-10 min. Then about 200 ml of stripping buffer was added to these blots in the plastic boxes and incubated in a shaking water bath at 50°C for 10 min. The blots were then transferred to new boxes and washed well with TBST for 1-2 h by changing TBST for every 20 min. The stripped blots were then incubated with blocking buffer followed by immunodetection as described earlier in this section.

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7 Appendix

7.1 Abbreviations

APS	Ammonium persulphate
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CO2	Carbon dioxide
Ctr	Chlamydia trachomatis
DNA	Deoxyribonucleic acid
e.g	For example (latin:'exempli gratia')
EB	Elementary body
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
GM	Growth medium
GO	Gene Ontology
h	Hour
H2O	Water
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIV	Human immunodeficiency virus
hpi	Hours post infection
HPV	Human papillomavirus
i.e.	That is (latin: 'id est')
IF	Immunofluorescence
IFU	Inclusion forming unit(s)
Ig	Immunoglobulin
IL8	Interleukin 8

IM	Infection medium
KD	Knockdown
l	Liter
LGV	Lymphogranuloma venereum
mA	Milliampere
MHC	Major histocompatibility complex
min	Minute
miRNA	MicroRNA
ml	Milliliter
mm	millimeter
mM	Millimolar
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
mRNA	MessengerRNA
NP40	Nonidet P-40
nt	Nucleotide
p.i	Post infection
PBS	Phosphate buffered saline
pH	Negative decadic logarithm of the hydrogen ion concentration (latin: 'potentia hydrogenii')
POC	Percent of control
PVDF	Polyvinylidene fluoride
RB	Reticulate body
RNAi	RNA interference
RNAi	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA

siRNA	Small interfering RNA
SPG	Sucrose–phosphate–glutamate
STD	Sexually transmitted diseases
TBE	Tris/Borate/EDTA
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with 0.5% Tween 20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TI	Transfection infection
TO	Transfection only
TTSS	Type three secretion system
UV	Ultraviolet
V	Volume
w/v	weight/volume
µl	Micro liter
µM	Micromolar

7.2 Erklärung

Hiermit erkläre ich, Rajendra Kumar Gurumurthy, geb. am 27.06.1977 in Oolavadi-KS, Indien, die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt zu haben und alle verwendeten Hilfsmittel und Inhalte aus anderen Quellen als solche kenntlich gemacht zu haben. Desweiteren versichere ich, dass die vorliegende Arbeit nie in dieser oder anderer Form Gegenstand eines früheren Promotionsverfahrens war. Die dem angestrebten Promotionsverfahren an der Mathematischen-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin zugrunde liegende Promotionsordnung ist mir bekannt.

Berlin, im June 2009 _____

Unterschrift

7.3 Acknowledgement

I express my sincere and heartfelt thanks to Prof. Thomas Meyer for providing me an opportunity to conduct my doctoral research in his lab. It makes me very fortunate to have worked under the supervision of such an experienced, encouraging, optimistic and supportive mentor. His generosity and commitment to his students is truly unmatched.

I am grateful to Dr. Simone Hess for always being there to discuss and give valuable advice when I needed them the most. I appreciate her confidence in me and the unwavering support at the beginning of my doctoral work at MPIIB. Her insightful comments and constructive criticism prevented me from going astray with my research projects.

I would like to convey my deep sense of gratitude and sincere thanks to Dr. Nikolaus Machuy for his generous help, expert advice, encouragement and constant support throughout my stay here. I am also thankful to Dr. André P. Mäurer for his incessant help and advice provided for my work. I am grateful to both of them for the assistance, careful reading and critical reviews of my scientific writings.

Many thanks go to colleagues and friends in the department of molecular biology, especially to the members of the Chlamydia research group at the MPI for Infection Biology, for their constant willingness for thorough scientific discussion and the nice working atmosphere. Special thanks go to Dr. Hesham Al-Younes, Dr. Dagmar Heuer for suggesting the controls for the screen. I also would like to thank Erik Gonzalez who helped me in the lab at times. My thanks are also due to Dr. Klaus-Peter Pleissner and Dr. Johannes Schuchhardt (Microdiscovery) for their help with the statistical analysis of the data. Some of the results shown here are the fruits of an amazing amount of work under strenuous conditions that could not have been accomplished without the team of highly skilled technicians Special thanks to Christiane Dimmler, Dominique Khalil and Jan-David Manntz. I thank you for your fantastic work and wish you the best.

No words seem strong enough to thank my wife Cindrilla Chumduri, she has been the single greatest motivation behind my research and a continuous source of encouragement. I owe my deepest gratitude to my parents for their enormous support, their strong belief in me and all my endeavours. With no regard for their personal health or comfort, they assured that I excel in all aspects of my life, and my education was no exception. Each achievement in my life is a tribute to their countless sacrifices. I am also thankful to my brother Raveendra, sisters Manjula and Veena as well as all my family members for being a constant source of love, concern and strength all these years.

Thanks to all fellow Indians in our Institute for help with everything, especially, Prashant, Arunava, Thulasi, Vibha, Ravi and Hakkim. Many thanks for the fantastic environment you people have provide for all those years. Last but not the least I thank all my teachers right from my primary school to my university without whom, I would not have come to this point.

7.4 Publications

Paland N, Böhme L, **Gurumurthy RK**, Mäurer A, Szczepek AJ, Rudel T (2008).Reduced display of tumor necrosis factor receptor I at the host cell surface supports infection with Chlamydia trachomatis. J Biol Chem. 2008 Mar 7; 283(10):6438-48. Epub 2007 Dec 31.

Rajendra Kumar Gurumurthy, André P. Mäurer, Nikolaus Machuy, Simone Hess, Klaus P. Pleissner, Johannes Schuchhardt, Thomas Rudel, Thomas F. Meyer. Loss of function screen identifies C. trachomatis induced dual regulation of Ras/Raf/Mek/Erk pathway through inactivation of Raf1. (Manuscript in preparation)